Microbiology of olives with emphasis on the antimicrobial activity of phenolic compounds

Tassou, Chrysoula Ch

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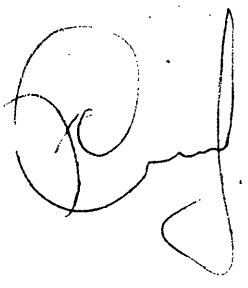
MICROBIOLOGY OF OLIVES WITH EMPHASIS ON THE ANTIMICROBIAL ACTIVITY OF PHENOLIC COMPOUNDS

Submitted by Chrysoula Ch. Tassou for the degree of Ph.D of the University of Bath 1993



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My family for their support

to George

CONTENTS

SUMMARY

Modern analytical procedures (HPLC) showed that olive fruits contain a range of phenolic compounds and that many of these are present also in virgin olive oil. The bitter taste of fresh (new) olive oil could be attributed to these substances also. The following compounds were identified: protocatechuic acid, tyrosol, hydroxytyrosol, caffeic acid, rutin, vanillic acid and ferulic acid. Oleuropein was identified in ethyl acetate extracts of green olives and, to a smaller extent, in black olives. Oleuropein was present in one of the virgin olive oil extracts also. Storage of either the olive oil or the phenolic extract thereof changed the HPLC profile.

The inhibitory effect of phenolic compounds and especially of oleuropein, on Gram negative and Gram positive bacteria common in food was exemplified by the results of this study. The effect of phenolic compounds and oleuropein on growth/survival of *Staphylococcus aureus, Salmonella enteritidis, Pseudomonas fragi, Enterococcus faecalis* were tested in broth and a model food system. It was found that growth and toxin production by *Staphylococcus aureus* was inhibited by both oleuropein and phenolics in general. Similar results were obtained with *Salmonella enteritidis*. In both cases the inoculum size, the concentration of inhibitor and the pH of the medium influenced inhibition. The germination of *Bacillus cereus* T spores was also inhibited by oleuropein. This inhibition was evident even when the oleuropein was added almost at the end of the phase bright to phase dark change.

The mode of action of phenolics could be attributed to their effect on the cell membrane. It was found that the concentration of proteins in a medium increased although there was no similar increase in cell production. Moreover electron microscopy revealed that the bacterial surface of the

vegetative cells of *Bacillus cereus* growing in a medium containing oleuropein became irregular and rough compared with cells growing in medium without the inhibitor. The inhibition by phenolics could not however be attributed to a specific compound. Oleuropein, tyrosol, rutin, vanillic acid were among the phenolics identified in extracts from olives and olive oil. The presence of these compounds in olive oil increased its antimicrobial activity against *Salmonella enteritidis* in mayonaisse.

Little attention has been given to the microorganisms on olives and their physiological activity, especially lipolysis. A survey was done in order to characterise some microbiological attributes of olives in orchards. Pseudomonads, yeasts and lactic acid bacteria were isolated. An unidentified *Pseudomonas* spp., *Aeromonas sobria, Pseudomonas putida*, and *Pseudomonas cepacia* were isolated most frequently. Eighty six percent of the pseudomonads and ninety one percent of the yeasts gave positive reaction, with tributyrin agar.

INTRODUCTION

The importance of olive cultivation and processing to the ecology and economy, both rural and national, of Greece is noted in Chapter 1. There are no reasons to believe that the prominence of the olive crop in the Greek economy will diminish in the near future. There are reasons, however, for anticipating a growing concern about the environmental impact of large scale production of edible olives and olive oil. Both commodities call for extensive use of water and both produce waste water that is not easily treated because of its high BOD_5 (25,000-90,000 mgO_2/l of water) and content of phenolic compounds having antimicrobial properties. One solution to the latter problem could well be the reclamation of such compounds from waste water and their use as preservatives.

The range and types of phenolic compounds in olive fruits and olive oils were explored with advanced analytical methods. The results are discussed in Chapter 2. The antimicrobial properties of oleuropein and phenolic substances extracted from olives were examined in detail in Chapter 3. It was demonstrated that these substances adversely affected the growth of bacteria, the germination of bacterial endospores and the production of extracellular substances by *Staphylococcus aureus*. Finally in Chapter 4 an overview of the microbiology of olives in the orchard is presented.

One notable feature of this thesis is the paltry number of citations of Greek studies on the various aspects of the fermentation of olive fruits and the production of olive oil. Much work in both areas has been done in the USA, Italy and elsewhere. In practice the present study has pulled together a diffuse literature and provided a foundation for future studies in Greece of an important agricultural crop and focused attention on the potential of phenols extracted from olives as preservative agents in food and feeds.

CHAPTER 1

THE OLIVE TREE AND ITS PRODUCTS

Introduction

The first reference to the olive tree is in the Holy Bible. In Genesis the flight of the pigeon with the olive branch announces the end of the flood. The olive is one of the most ancient of cultivated trees especially in the Mediterranean basin. Judging from excavations in the island of Crete, the culture of olive trees in Greece was practised as early as 2000-1500 B.C. According to Greek mythology, the city of Athens was founded by Cecropus and endowed with an olive tree by Minerva, the godess of wisdom (Balatsouras 1972). The island of Samos was given its name by Aeschylus because of the cultivation of olive trees and Thales of Miletus (639-546 B.C.) mentions that the two Aegian islands, Chios and Delos, were noted for olive oil production. It is mentioned in the Iliad of Homer that olive culture was known and practised in the islands of the Ionian Sea, especially in Corfu and Ithaca. In the books of Herodotus it is stated that the island of Eubea was covered by olive trees whereas they were unknown in Babylonia and Irak. Olives were of great importance to the ancient Greeks. They were an important item of diet and used in religion, in the decoration of pots, walls, gold and many other objects. Additionaly it was the symbol of peace, wisdom and victory. A wreath of wild olive branches was offered to the winners in the Olympic Games.

Olive tree cultivation

During the 15th century Spanish colonizers and Franciscan monks

extended the planting of the tree and the processing of table olives to various parts of the New World. Plantations in California were established in the 18th century. Initially olives for oil production were planted but later edible varieties were grafted on to old stock. Italian and Spanish migrants and missionaries took the olive tree to Australia, South Africa and Japan. The history of the spread of olive tree plantations has been described in detail (Kalogueria 1932; Molinari & Nicolea 1974; Garoglio 1950; Hartmann 1953; Patac *et al.*, 1954; Foytic 1960; Goor 1966). As will be evident below a great many of the studies of the science and technology of olive fruit processing were done in the New World rather than in Greece.

Olives are now distributed approximately between the latitudes 25° to 45° North and 15° to 35° South. Today over 800 million olive trees are grown on almost 10 million hectares throughout the world. According to the International Olive Oil Council (Anon. 1984), almost 98% of olive cultivation is concentrated in the Mediterranean countries. Spain and Italy together have 40% of olive cultivation and 50% of olive production (Table 1.1). Greece is in second place in edible olive production after Spain and third in olive oil production (16% of the world production). Olives are cultivated on 14.1% of the cultivated land in Greece.

Agricultural and ecological aspects related to the olive tree

Olive farming plays a very important economic, social and ecological role in the Mediterranean countries. The value of the crop represents a very high percentage of the national agricultural income. As many of the European countries noted in Table 1.1 have little industry, olive production represents a substantial part of the gross national product. Many of the olive-producing countries in the Mediterranean basin export a considerable proportion of their products, thus assisting the balance of trade (Lombardo 1988). The olive tree helps to keep an ecological balance. When it is located in "fragile" areas

Table 1.1. Geographic distribution of olive production

Europe 84%*	Asia 5.5%*	Africa 8.5%*	America 2%*
Spain 42%	Turkey 3%	Tunisia 5%	Argentina 1.9%
Italy 24%	Syria	Algeria	USA
Greece 12%	Lebanon	Morocco	Mexico
Portugal 6.6%	Israel	Libya	Uruguay
Cyprus	Jordan	Egypt	Chile
France	Iraq	S.Africa	Peru
USSR	Iran		
Yugoslavia	Japan		

* percentage of world production
Based on Fedeli (1977)

-fragile from the point of view of erosion etc- it helps to conserve the soil without any continuing economic cost. As for the social aspects, most of the olive-growing areas are also the least-favoured regions of the EEC, their levels of income being among the lowest in the Community. Moreover these areas have little potential for the production of other crops because of the poor soil. Olive farming, which generates a considerable amount of employment, is therefore crucial in keeping the people on the land (Lapatas 1988).

The olive is a relatively small evergreen tree with narrow, silvery leaves and small white flowers, notable for its longevity. The original species of the current cultivars is unknown. It is considered by some to be a species of *Oleaster*, the wild type of which exists currently in North Africa, Portugal, Southern France, Italy and near the Black Sea. Another species, *Olea chrysophylla*, has also been considered to be the origin of current cultivars (Standish 1960). The official name of the olive tree grown today, *Olea europaea*, comes from Greek. It belongs to the family *Oleaceae* which comprises about 30 species in several genera.

The olive thrives in deep, easily drained soils and in clear, dry atmospheres with moderate temperatures. The last mentioned should never fall below 15° F (-9°C) if fruit is to be produced. A certain degree of chilling, differing considerably among cultivars, is necessary however for the initiation of flowering (Hartmann & Prolingis 1957). Olive production often follows a 2-year cycle, with a good crop one year followed by a medium or poor one the next. Such a pattern creates serious microbiological problems for the industry involved with olive products. The causes of alternate year fruit-bearing, as well as poor fruit production in general, include insufficient irrigation, inadequate fertilization, excessively dense planting, lack of adequate pest and disease control and the use of a single cultivar over a wide

area. The last feature reduces the chances of cross-pollination (Hartmann & Bougas 1970).

When olives are stored in heaps several chemical and biochemical changes occur. Perhaps the most damaging but least explored one is caused by the so-called "fermentation of olives". This is a term describing the reactions caused by enzymes endogenous to the olive tissue and those produced by bacteria, yeasts and moulds growing on it. The activity of certain endogenous enzymes (eg. lipolytic, pectinolytic) in the unripe olive fruit is minimal, but in the ripe fruit their activity becomes significant (Suarez-Martinez 1975). As the olive tissue respires heat is produced which, if not effectively dissipated from the heaps of olives, accelerates enzymatic reactions. Lipolysis, lipid oxidation and other undesirable reactions occur leading to a lowering of the quality of olives and olive oil. Fungi, which can grow on olives in store may produce aflatoxins which may be carried over in the extracted oil (Gracian & Arevalo 1980, Tantaoui-Elaraki *et al.* 1983a,b). The microbiology of heaps of olives is discussed on pp. 165.

Olive products

The major products of the olive industry are listed in Table 1.2. The world's olive production is 8-9 million tonnes, of which 400-700 thousands tonnes are used for edible olives and the rest for oil production.

Fermented olives

Small quantities of Californian ripe olives picked directly from the tree - Californian green-ripe olives - are consumed immediately after harvest and Greek-type salt-cured olives, which are dehydrated and preserved in large quantities of dry salt, are the only ones produced without a lactic acid fermentation. All other types of olives undergo at least some lactic acid

Table 1.2. Principal olive products on the world market

A. EDIBLE OLIVES	
1. Fermented olives	2. Non-fermented olives
a. Spanish-style green	a. Californian green-ripe
b. Pickled black	b. Salt-cured Greek type
c. Greek-type brined	
B. OLIVE OIL	
1. Virgin olive oil	
2. Refined olive oil	
3. Pure olive oil	
4. Residual or husk oil	
5. Oil of endocarps	

Based on Fernandez Diez (1971), Fedeli (1977), Fernandez Diez (1983), Kiritsakis & Markakis (1987).

fermentation during storage in brines.

Indeed fermentation is without doubt the most important process applied to olives before entering national/international markets (Fernandez Diez 1971; Vaughn 1982). With the Spanish-style green-fermented olives the fruits are harvested whilst still yellow or a greenish-yellow. They are treated with sodium hydroxide (1.3 or 2.6% w/v) for 6-10 h. This eliminates the greater part of the bitter agent, oleuropein (see Chapter 2) as well as compounds that inhibit microbial fermentation (see Chapter 3). The fruits are then washed to eliminate the major portion of the lye. Some of the sugars are removed with the wash water also. The olives are then placed in sodium chloride (9-11 Be; degrees areometer Baume) in which a lactic acid fermentation takes place.

With pickled black olives the fruit is treated successively with sodium hydroxide (1-2% w/v) solutions to achieve a progressive penetration of lye into the pulp. After each alkaline treatment, the olives are placed in water and oxidized by injecting air. The consequent oxidation of the polyphenolic compounds ensures a complete blackening of the skin of the fruit and a uniform colouration of the pulp. After lye treatment, washing and oxidation, the olives are washed several times with water to remove most of the sodium hydroxide. Generally, iron gluconate (0.1% w/v) is added to the last wash to stabilize the colour achieved by oxidation. The olives are then placed in a brine (3% w/v sodium chloride) and finally packed in glass jars or tin cans (Fernandez Diez 1983).

Brined or wet-processed Greek-type olives are usually prepared from firm but highly coloured (purple to jet-black) fruit. The olives are sorted and graded for size, placed in barrels and covered with brine (7-10% w/v NaCl). The salt content of the brine is then increased until it is *ca* 15% (w/v) or more. The olives cure slowly, lose some of their natural bitterness and take

on a purple colour. The fermentation process is very feeble because diffusion of soluble components through the skin is slow when it has not been treated with alkali (Balatsouras 1972).

Fermentation

The origin of the art of preparing table olives by lactic acid fermentation is lost in antiquity and our understanding of the biochemical and microbiological aspects of the fermentation is of comparatively recent origin. The role of lactic acid fermentation in the preservation of table olives was recognised during the last quarter of the 19th century (Vaughn 1975). Some of the following reasons may explain why lactic acid fermentation has maintained its popularity throughout the centuries:

a) It provides a means of storage whereby a commodity can be preserved without seriously impairing nutritional and physical properties.

b) It produces an acidic product that is unlikely to support the growth of microorganisms of public health significance.

c) It creates products that possess unique and distinctive flavours.

d) It can be done with commonly available equipment, such as barrels, with low capital and running costs. Consequently the process can be done by both the home and small-scale commercial food producer (Stamer 1988).

Microbiology of fermentation

In normal fermentations of olives, three stages in the selection of microorganisms are recognised: primary, intermediate and final (Table 1.3).

The primary stage

According to Pelagatti (1978-80) -see Table 4.1- a diverse range of

Table 1.3. Microorganisms which contribute to olive fermentation or spoilage

Fermentative microorganisms	Spoilage microorganisms
Primary stage	
Streptococcus spp.	*Aerobacter* spp.
Pediococcus spp.	*Escherichia* spp.
Leuconostoc mesenteroides	*"Paracolobacterium"*
Leuconostoc dextranicum	*Aeromonas* spp.
	"Achromobacter"
	Clostridium spp.
	Bacillus polymyxa
	Bacillus macerans
	Yeasts
Intermediate stage	
Leuconostoc mesenteroides	
Lactobacillus plantarum	
Final stage	
Lactobacillus brevis	*Propionibacterium* spp.
Lactobacillus buchneri	*Candida* spp.
Lactobacillus plantarum	*Pichia* spp.
	Rhodotorula spp.
	Micro-fungi

Based on Vaughn *et al.* (1943, 1969a,b)

Lactobacillus spp. are present on green olives picked from the tree. Only small numbers of lactic acid bacteria, for the most part low-acid tolerant representatives of the genera *Streptococcus, Pediococcus* and *Leuconostoc* and very few *Lactobacillus,* are recovered during the initial phase. *Leuconostoc mesenteroides* dominates the latter phase of primary fermentation as well as the early part of the intermediate stage. According to Vaughn *et al.* (1943) *Leuconostoc dextranicum* is encountered infrequently.

If, however, the fermentation does not start normally, then undesirable microorganisms may predominate and spoil the olives at the outset. Most of the spoilage at this stage is caused by Gram-negative bacteria. If not controlled, such organisms produce gas pockets in the olive as a consequence of fermentation, and pectinolytic ones soften the tissue. Coliforms such as *Aerobacter, Escherichia* & "*Paracolobacterium*", as well as pseudomonads, *Aeromonas* and "*Achromobacter*" (West *et al.* 1941; Vaughn *et al.* 1969b) have been associated with such problems (Table 1.3). Gram-positive bacteria may also be a problem. Species of *Clostridium* cause a butyric acid fermentation and the formation of gas pockets in olives (Gilliland & Vaughn 1943). Members of the genus *Bacillus* have been implicated in softening of olives (Nortje & Vaughn 1953) and the *Bacillus polymyxa-macerans* group also causes gas-pocket formation. Yeasts also play a role in competing for the fermentable material and have been known to cause "stuck" fermentations - ethanol rather than lactic acid being the principal product of fermentation (Vaughn *et al.* 1943).

The intermediate stage

Lactic acid bacteria predominate in the intermediate phase of a normal fermentation. They include a mixture of the low-acid tolerant species of *Leuconostoc* and the highly-acid tolerant members of the genus *Lactobacillus*. *Lactobacillus plantarum* dominates the latter phase of the intermediate as

well as the final stage of fermentation. A blemish of olives known as "yeast spots" is caused by *Lact.plantarum* forming colonies beneath the stomatal openings in the skin of olives (Vaughn *et al.* 1953). With a normal fermentation the Gram-negative bacteria disappear completely by 12-15 days.

The final stage

Apart from the numerically dominant *Lact.plantarum*, *Lactobacillus brevis*, the most commonly occuring of the heterofermentative species of this genus, has been found in the latter phases of the intermediate as well as during the final stage of fermentation. Two other heterofermentative species, *Lact.buchneri* and *Lact.fermentum*, have been encountered infrequently. Small numbers of yeasts may persist throughout a properly controlled fermentation but cause no problems if the contents of the container are kept anaerobic.

It is essential that the fermentation produces enough total acidity to decrease the pH of the brine to at least 4.0 and preferably to 3.8, otherwise the olives will be liable to "zapatera" spoilage. This malodorous condition develops in olives when the fermentation does not produce enough total acidity to decrease the pH of the brine below 4.5. At this pH value and when fermentable material is no longer available for the lactic acid bacteria, the lactic and acetic acids in the brine serve as energy sources for spoilage bacteria. It has been shown that spoiled brines contain formic, propionic, butyric, valeric, caproic and caprylic acids (Delmouzos *et al.* 1953). The off-odour at first was described as cheesy. Species of *Propionibacterium* have been found to use the lactic acid in brines to produce the propionic acid responsible for this odour (Plastourgos & Vaughn 1957). Species of *Clostridium* convert lactic and acetic acids to the malodorous higher volatile acids responsible for the foul odours in affected brines (Kawatomari & Vaughn 1956).

During fermentation and subsequent-storage of olives, a yeast flora develops even in completely filled barrels and in vats with a surface covering. Species of *Candida* and *Pichia* have been identified (Mrak *et al.* 1956). Some pectinolytic pink yeasts of the genus *Rhodotorula* isolated from brines were shown to be associated with softening of olives (Vaughn *et al.* 1969a). Some fungi, along with bacteria and yeasts which form a film on the surface of the storage tanks, may produce pectinases and cause softening of stored olives (Balatsouras & Vaughn 1958).

Control of the fermentation

As faulty fermentation of olives has been an important problem for many years, some investigators have recommended various solutions *viz*: a) incubation at a temperature (*ca.* 21-24°C) that accelerates lactic acid production (Cruess 1930); b) addition of supplementary fermentable material such as sugars and c) the addition of starter cultures - the use of pure cultures of lactic acid bacteria (for example *Lact. plantarum*) to initiate the fermentation. The addition of glucose stimulates acid production and ensures a decrease in the pH to a level (3.8-4.0) which is ideal for the growth of lactic acid bacteria. Maintenance of anaerobic or nearly anaerobic conditions to suppress oxidative yeasts and moulds is another important aspect of the fermentation of olives. It is necessary also to adjust the salt concentration of the brine to 5-7 NaCl g/100 ml of water (Vaughn 1954).

Samish *et al.* (1966, 1968) noted a remarkable increase in the rate of lactic acid fermentation of olives which, following processing with lye to remove the bitter flavour, had been given a short heat treatment in water. They suggested that this probably destroyed or attenuated a naturally occurring inhibitory substance in the olive flesh. Through damaging the cell membranes, this treatment may enhance also the diffusion of growth substances to the brine. In practice the fermentability of different varieties of

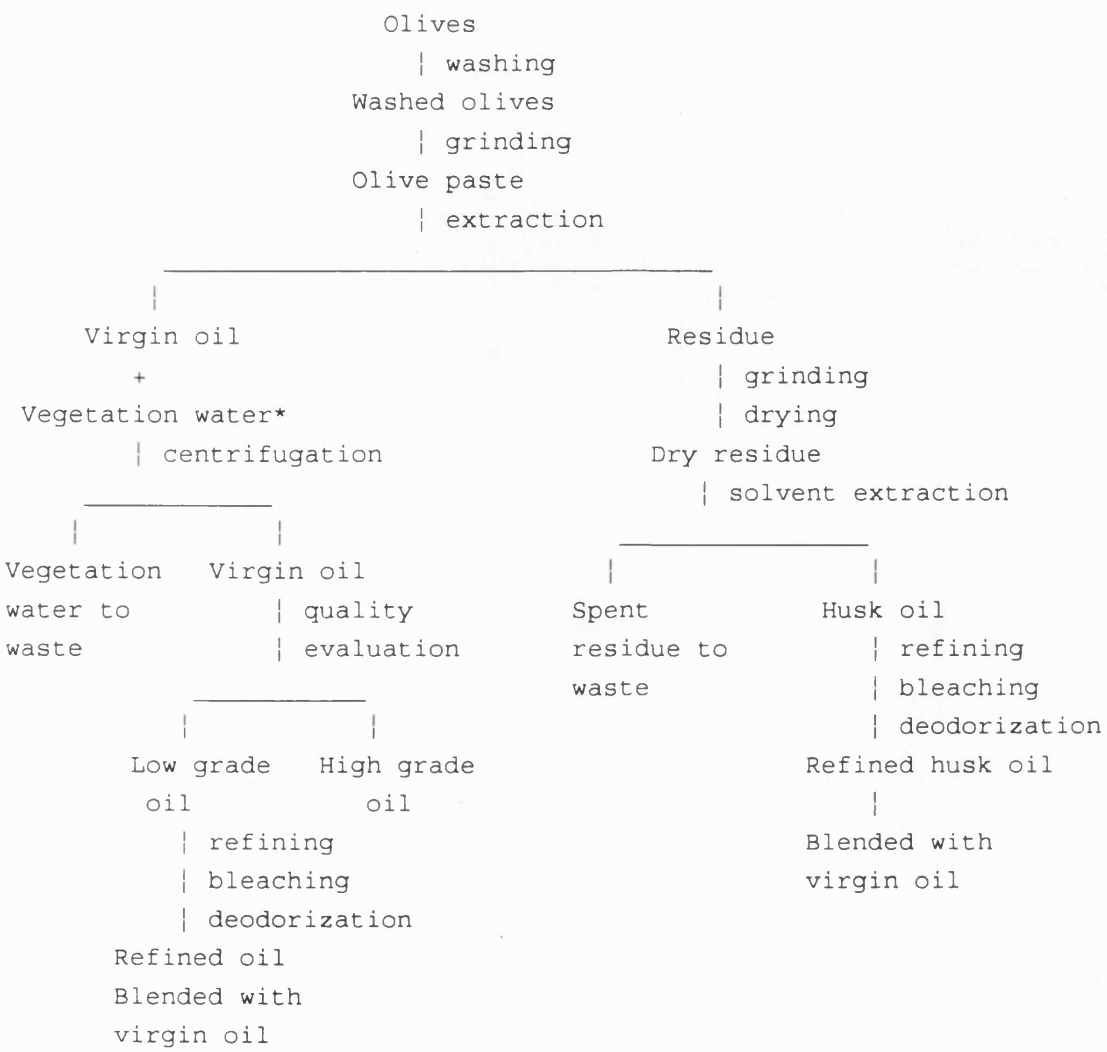
olives depends more on their content of inhibitors than on any other single property. Borbolla y Alcala *et al.* (1969) surmised that treatment with an adequate concentration of lye at appropriate temperatures destroys the antibiotic substance in olives in the same manner as the heat treatment. Pretreatment of the olives in 1.8% (w/v) NaOH solution in order to remove the bitter oleuropein has the added advantage of increasing the salt diffusivity almost 5-fold. Very recently Ruiz Barba *et al.* (1993) suggested that the increased permeabilities of fruit to polyphenols and organic compounds are probably the primary cause of the rapid fermentation of alkaline-treated, but not of untreated olives and that it could well be that the balance of inhibitory polyphenols/nutrients and neutralizing compounds determines whether or not an olive brine is likely to ferment properly.

The preparative stages of olive fermentation produce waste water that is not easily treated by the normally used biological systems. The possible use of oleuropein-splitting yeasts (*Candida veronae*) in the microbiological debittering of table olives was suggested by Balloni *et al.* (1977) and Pelagatti (1981-1983). This would ameliorate the problems of waste water disposal. Olives prepared with an entirely microbiological process showed distinctly different chemical attributes and had a better taste and flavour compared with those treated with caustic soda. The latter causes chemical modifications in the fruit as well as the loss of many soluble constituents that contribute to the desirable properties of the best quality table olives.

Olive oil

Ideally the extraction of oil should follow harvesting without delay because of problems stemming from "fermentation of olives" that were discussed above. Figure 1.1 shows the various steps during the extraction process. In olive oil mills the extraction is achieved mechanically. The oil

Fig. 1.1. Olive oil extraction



*: Vegetation water is the water content of olives which is separated from the oil by centrifugation

Based on Fedeli (1977)

obtained without any additional processing is termed, virgin olive oil. The refined olive oil has been neutralized, deodorized and bleached. The pure olive oil is a blend of virgin and refined oil and residual or husk oil is that obtained from the residue olive paste with solvent extraction. Over the past few years efforts have been made to increase the yield of oil from olives by the use of enzymes. Pectin depolymerase, papain, cellulase, hemicellulase and an acid protease as well as polygalacturonase have been shown to increase oil yield and/or decrease the extraction time (Montedoro 1972, 1987; Montedoro & Petruccioli 1978; Leone *et al.* 1977; Santos Antuenes 1978). Simultaneous use of enzymes and absorbing agents such as Polyclar AT, methyl-cellulose, albumin for tannin phenolics was reported to have increased the yield and improved the quality of olive oil (Montedoro & Petruccioli 1974; Montedoro *et al.* 1976).

It is evident from the above discussion that the preparative phase of olive fermentation poses problems. It uses much water and produces waste water that is not easily treated because of its content of NaOH, NaCl, antimicrobial agents and high BOD_5 a topic discussed in Chapter 3. A possible solution to these problems would be the reclamation of the antimicrobial agents for possible use as preservatives in food. The potential of such agents in food preservation was examined in this study (Chapter 3).

CHAPTER 2

CHEMICAL COMPOSITION OF OLIVE FRUITS

The olive fruit

The olive fruit (Fig. 2.1) is an oval drupe akin to peaches, cherries etc. It consists of the pericarp and the endocarp (nucleus). The pericarp comprises: a) the epicarp (skin), which is 1.5-3.5% of the weight of the fruit, b) the mesocarp (or flesh), a tissue rich in oil and water, comprises 66-85% of the fruit, and c) the endocarp (or pit) -comprising 10-30% of fruit weight- is a hard woody part containing one or rarely two seeds (Kiritsakis 1988).

According to Fedeli (1977) the average chemical composition (% w/w) of the olive fruit is: water 50.0; oil 22.0; carbohydrates 19.1; proteins 1.6; cellulose 5.8 and ash 1.5. The chemical composition of the mesocarp of three Greek varieties of olives is shown in Table 2.1. With the olive fruit, the edible portion consists of the skin and the flesh. Both of these are built up of parenchymatous cells which in a cross-section appear as in Fig. 2.1. The fleshy mesocarp is bitter when harvested such that olives are inedible. There are two main causes of bitterness, (1) lack of sugar in concentrations sufficient to impart sweetness, and (2) the presence, especially in the mesocarp, of a bitter agent, oleuropein.

For the most part water is confined to the cell vacuole (Fig. 2.1) and to a lesser extent the cytoplasm and the cell wall. The vacuole "sap" consists of water-soluble substances such as simple "sugars" (mannitol, hexoses, sucrose etc.), organic acids, tannins, oleuropein, minerals etc. (Balatsouras 1972).

Fatty substances, exclusively constituents of the cytoplasm, occur in most drupes at a percentage as low as 1.5 % (w/w). These occur mainly in the form of lipoproteins. Exceptionally in the olive and avocado, the fatty substances occur in the form of small, distinct drops which account, at least

Fig. 2.1a. Parts of the olive fruit

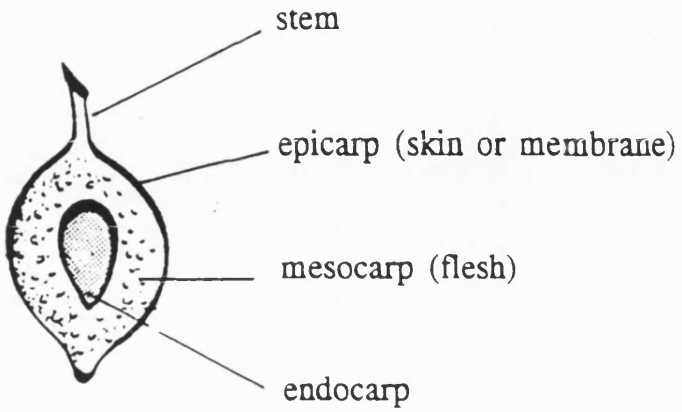


Fig. 2.1b. Cross-section of parenchymatous cells

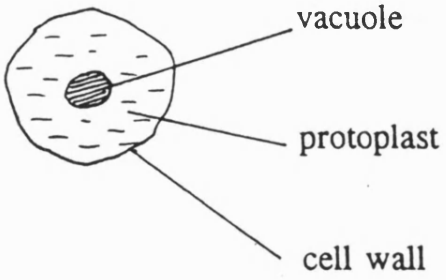


Table 2.1. Composition of the mesocarp of three Greek varieties of olives

	Varieties		
Constituents	Koroneiki#	Throumpolia$	Megaritiki$
Water	72.5*	63.3	64.4
Fatty substances	16.8	30.2	21.8
Ash	1.2	1.2	1.6
Nitrogen (total)	0.3	0.2	0.3
Nitrogen (free)	0.003	-	-
Carbohydrates	9.2	5.1	11.9

* : g/100g fresh weight
# : variety used mainly for olive oil production
$: varieties used mainly for edible olive production

Based on Manoukas (1972)

in the case of olives, for 20-35 % (w/w) of the mesocarp weight. Other fatty substances are constituents of the wax-like material that impregnates and thereby protects the epidermis from water. One of these, cutin, is a polymeric compound composed of aromatic units, the actual counterpart of subecin that impregnates the corky cells (Balatsouras 1972).

In the Mediterranean area there is a low oil content in the fruit in June followed by a progressive increase in the flesh and thereafter in the endocarp in July (Kiritsakis 1988). Oil content is important both for the textural characteristics and the nutritive value of processed olives. The fact that ripe olives contain more oil than the green ones is of significance due to the fact that the former are used as a bulk food and the latter as an appetizer (Balatsouras 1972).

Oil formation

Although oil formation in the olive fruit has been investigated since the last century, only recently has its synthesis been elucidated. Thus Hess (1975) concluded that the biosynthesis of the triglycerides involves three processes: a) Fatty acids are synthesized by successive addition of malonyl-CoA to a primer molecule of acetyl-CoA. As the three-carbon malonyl residue is decarboxylated after each addition, the progressive lengthening of the fatty acid chain by two carbon atoms is understandable. A multienzyme system catalyzes the condensation, reduction and dehydration reactions that are necessary for the completion of fatty acid synthesis. b) Glycerol phosphate is formed from the dihydroxy- acetone-phosphate of the glycolytic pathway. c) Fatty acids, as CoA derivatives, are then transferred to the free hydroxy groups of the glycerol phosphate. Dephosphorylation and completion of the esterification of glycerol follows.

Glucose, fructose, galactose, arabinose and sucrose are the major soluble carbohydrates in olive fruits (Ragazzi & Veronese 1967; Fernandez Diez *et al.* 1981; Fernandez Bolanos *et al.* 1982). Small amounts of xylose and rhamnose are present also (Table 2.2). Mannitol, approximate range of 0.5-1% (w/w) of the fresh pulp, has also been found (Borbolla y Alcala *et al.* 1956). The carbohydrate content is of primary importance due to the fact that it affects the fermentability of olives in the sense that the more carbohydrates the easier the fermentation and the greater the amount of lactic acid formed to aid the preservation. Besides water soluble-sugars, polymers such as cellulose, hemicelluloses and small quantities of pectic substances are present mainly in the cell wall (Heredia Moreno 1979; 1980a,b). As cellulose does not increase in amount with ripening, ripe olives are not tough and woody.

The pectins are not strictly speaking polysaccharides but straight-chained galacturonic acid residues linked through 1,4-α-glycosidic bonds. Some of the free carboxyls are esterified with methyl-alcohol and the chains are randomly cross-linked by bivalent cations and hydrogen bonds. Residues of simple sugars are claimed to participate as building blocks of the whole molecule. Pectic substances, which represent about 0.3-0.6% (w/w) of the weight of the fresh-pulp, are of great importance in the texture of the fruit. These change markedly with the progress of fruit development. This change is reflected in the structural characteristics of the fruit. Pectic substances of olive fruit are subject to hydrolysis by pectinolytic enzymes elaborated by various microorganisms. When pectic material of olive tissues breaks down, the flesh disintegrates completely. Such breakdown ruins edible olives as well as the quality of fermented stock (Chung *et al.* 1974; Fernandez Diez *et al.* 1974; Minguez Mosquera *et al.* 1976).

Proteins are present in the olive fruit at approximately 1.5% (w/w). They

Table 2.2. Constituents of olive fruit

Pigments	*Amounts*[&]
chlorophyll α	43.35
chlorophyll β	9.73
β-carotene	2.36
lutein	4.30
luteoxanthine	-
violoxanthine	2.23
neoxanthine	1.61
phytofluene	
ζ-carotene	
cyanidin-3-glucoside	
cyanidin-3-rutinoside	

Organic acids	*Amounts*[#]
citric	0.1 - 0.15%
malic	0.01 - 0.07%
oxalic	0.1 - 0.17%
fumaric	
tartaric	
lactic	
acetic	

Sugars[*]
glucose
fructose
galactose
arabinose
sucrose
xylose
rhamnose
mannitol

&: mg/Kg in fresh destoned olives
#: w/w in fresh olives
*: no concentration has been reported

Based on Ragazzi & Veronese (1967), Ragazzi *et al.* (1967), Manoukas (1972), Vazquez-Roncero *et al.* (1974), Fedeli (1977), Fernandez Diez *et al.* (1981), Aligizakis (1982), Fernandez Bolanos *et al.* (1982), Kiritsakis (1988), Minguez Mosquera *et al.* (1988, 1990).

are the main constituents of the jelly-like cytoplasm and also of cytoplasmic membranes that confer selective permeability to the cell. These proteins contain all the basic amino-acids which exist in the other plant tissues. The following are those in relatively high amounts: arginine, aspartic acid, glutamic acid and to less extent, leucine and valine (Table 2.3a; Manoukas 1972).

Tannins are present in relatively high concentrations - up to 1.5-2% (w/w) of the mesocarp weight. They are responsible for the astringency of raw olives. A tannin-like substance, oleuropein, occurs exclusively in the olive fruit and causes many problems in processing (discussed in Chapter 3).

The pigments (Table 2.2) are significant in ripe olives. Both chlorophylls α and β as well as carotenoids occur in the ratio of 2.5 to 1 in olives. Both chlorophylls and carotenoids, lipid-soluble substances, are important in imparting colour to green olives. The lactic fermentation causes total transformation of the chlorophylls present in the fresh fruit by two mechanisms. One is enzymatic, due to the action of chlorophyllase. The other is caused by the acid pH of the medium (Minguez Mosquera *et al.* 1990). Besides the fat-soluble colouring agents, anthocyanins are formed during ripening of the fruit. These water-soluble substances are formed in the cell sap, being synthesized progressively during ripening and accumulating mainly in the epidermal cells. Anthocyanins occur in the flesh of some olive varieties. This characteristic is of particular importance for processing of naturally ripe fruit (Balatsouras 1972).

Organic acids and their salts are present in the juice of the fruit in concentrations of 0.5-1 % (w/w). The acids present in olives are: citric, malic, oxalic, malonic, fumaric, tartaric, lactic, acetic (Table 2.2). They are of importance during the fermentation of the commodity because of their

Table 2.3. (a) Amino acids in olive fruits and (b) Mineral constituents present in the olive pulp of a Greek cultivar, Megaritiki

a.

Constituent	Content	Constituent	Content
Glutamic acid	372[$]	Alanine	149
Aspartic acid	316	Phenylalanine	124
Leucine	248	Arginine	113
Proline	238	Tyrosine	69
Valine	165	Lysine	49
Glycine	157	Istidine	29
Isoleucin	150	Methionine	13
Threonine	123	Cystine-cysteine	2
Serine	114		

b.

Calcium	386[*]	Phosphorus	188
Chlorine	683	Potassium	3642
Iron	14	Sodium	48
Magnesium	192	Zinc	7
Manganese	2		

a : Based on Balloni *et al.* (1977)
b : Based on Manoukas (1972)
$: mg/100g dry pulp
* : ppm in raw pulp

buffering capacity (Fernandez Diez & Gonzalez Pellisso 1956).

The minerals (Table 2.3b) are dissolved mainly in the cell sap but they occur also, combined with other cell constituents, in the cytoplasm as well as in combination with pectic substances in the cell wall (Balatsouras 1972).

Phenolic compounds

Information about phenolic substances is concerned mainly with fermented olives and olive oil. The fact that these substances play an important role in olive fermentation (see Chapter 3) and are transferred to the extracted olive oil is of great practical significance.

In general total polyphenols account for 7% (w/w) of dry matter (expressed as caffeic acid) in green but diminishes to about a half of the original concentration in ripe fruit (Vazquez Roncero *et al.* 1971). The content of phenolic components is 1-3% (w/w expressed as tannic acid) in ripe fresh pulp. A variety of phenolic components have been found, caffeic and ferulic acid being among the simpler of these (Table 2.4). Considerable differences have been observed in phenolic compounds in different olive varieties and at different stages of maturation (Amiot *et al.* 1986). The tannins and anthocyanins are of great importance in the taste and appearance of edible olives and olive oil. Anthocyanins, especially the glucosides of cyanidin and peonidin, are responsible for the purple and blue colour of ripe olives. Phenolic substances are also responsible for the browning rate in olives (Sciancalepore 1985).

The type and the amount of phenolic substances in olive oil has been established (Kiritsakis 1988, Papadopoulos & Tsimidou 1992, Tsimidou *et al.* 1992a). Their presence is very important because as antioxidants they contribute to the stability and hence shelf life of olive oil (Chimi *et al.* 1988, Papadopoulos & Boskou 1991, Tsimidou *et al.* 1992b). Indeed it is well

Table 2.4. Phenolic compounds in olive fruit

Oleuropein
Dimethyloleuropein
Luteoline-5-glucoside
Tyrosol
Hydroxytyrosol
Caffeic acid
Protocatechuic acid
p-coumaric acid
Gallic acid
Ferulic acid
Vanillic acid
Dihydrocaffeic acid
Quercetin-3-rutinoside (rutin)
Luteoline-5-glucoside
Apigenin-7-glucoside
Verbascoside
Ligstroside
Oleoside
3-methoxybenzoic acid
4-hydroxybenzoic acid
4-hydroxycinnamic acid
3,4,5-trimethoxybenzoic acid
3,4,5-trihydroxyphenylacetic

Based on Ragazzi & Veronese (1967), Vazquez Roncero *et al.* (1974), Fedeli & Camurati (1974), Fedeli (1977), Balatsouras (1982), Fleuriet *et al.* (1984), Amiot *et al.* (1986), Gariboldi *et al.* (1986).

documented that the storage life of olive oil is longer than that of other vegetable oils mainly due to the former's greater content of phenolic compounds. It is well known that phenolic acids and their derivatives are significant components of the taste and odour profile of some plant products (Torres *et al.* 1987) such as tea or coffee (Bailey *et al.* 1990) and especially olives (Cruess & Alsberg 1934) and products thereof (Vazquez Roncero 1978, Drusas *et al.* 1988). Indeed in the case of olive oil the bitter taste has been attributed to phenolics (Gutierrez *et al.* 1989). Of the phenolics listed above, oleuropein is discussed further because of its potential use as an antimicrobial agent.

Oleuropein

Oleuropein, a phenolic glucoside typical of olives, is responsible for the bitter taste of immature fruit (Cruess & Alsberg 1934; Shasha & Leibowitz 1959a,b, 1961; Cohen & Lifshitz 1969; Cohen *et al.* 1967) and for the inhibition/delay of fermentation of green olives (Fernadez-Diez 1983). Additionally, according to Simpson *et al.* (1961), the colour of black olives is due to the oxidation products of oleuropein and other phenolics.

Oleuropein and its hydrolysis products have been isolated using a variety of solvents (Table 2.5) along with other phenolic substances such as caffeic acid, vanillic acid, syringic acid, catechol etc. not only from olive pulp remaining after oil extraction (Vazquez-Roncero *et al.* 1974), but also from the water used to wash olive paste during oil extraction (Ragazzi & Veronese 1967; Balice & Cera 1984). The bitter principle of olives was first studied and named oleuropein by Bourquelot and Vintilesco (1908) who found it in fruit, leaves and bark. They described it as a non-crystalline, intensely bitter glucoside, readily soluble in alcohol, fairly soluble in water and practically insoluble in ether. Its specific rotation was given as -1270 and its reducing power on Fehling's solution as 0.412 that of dextrose. Subsequently many

Table 2.5. Solvents used for the extraction of phenolic compounds from various parts of the olive tree (*Olea europaea*) as well as from different stages of oil extraction

Solvent	Parts used
Ethyl acetate	green olives & waste waters from olive oil mills
Methanol	green olives, bark, waste water from olive oil mills, leaves, stems
Methanol/ chloroform	olive oil, olives and leaves
Ethanol	green olives
Acetone	leaves
n-propanol	waste water from olive oil mills

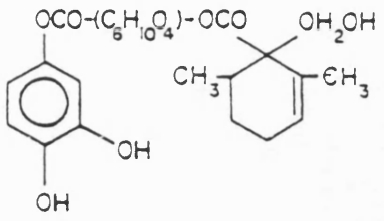
Based on Panizzi *et al.* (1960), Ragazzi & Veronese (1967), Vazquez-Roncero *et al.* (1974), Rodriguez *et al.* (1988)

investigators attempted to isolate oleuropein and to describe its structure and properties.

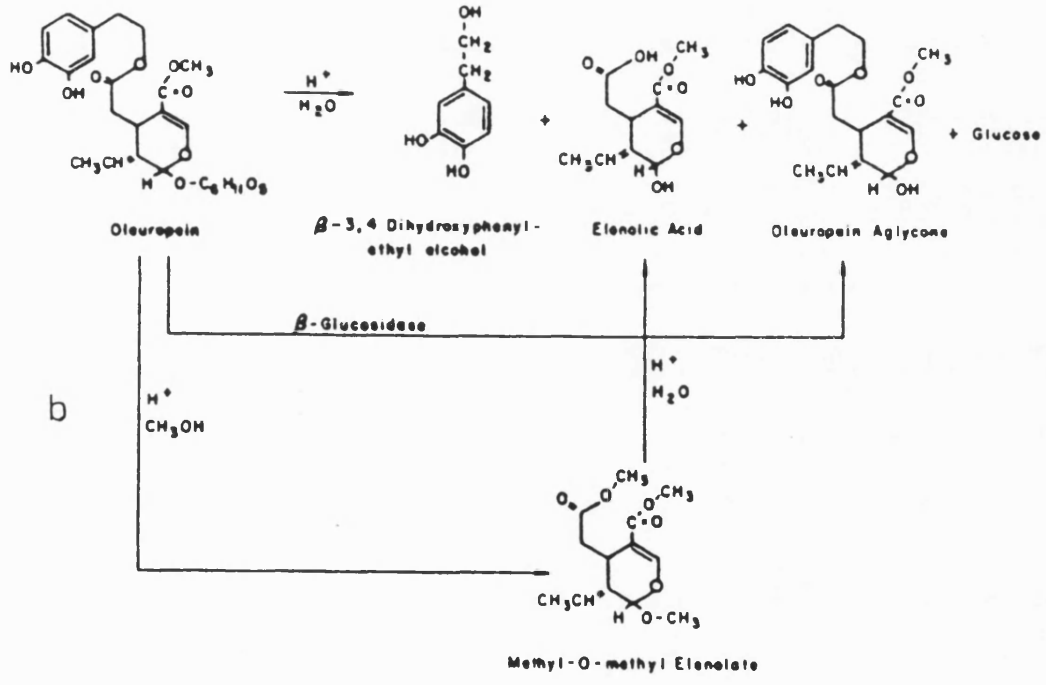
Power and Tutin (1908a,b,c) prepared a bitter mixture of several amorphous compounds from olive leaves and bark by the method of Bourquelot and Vintilesco. They inferred that "the oleuropein" of the latter investigators was not a pure substance. Vanzetti (1909) obtained a bitter amorphous substance from olive twigs and Hilts and Hollingshead (1920) suggested that the bitter principle of olives was a tannin-like substance. Cruess and Alsberg (1934) confirmed that oleuropein shared common characteristics with tannin. Their preparations reduced a potassium permanganate solution and were hydrolyzed both by acid and Pectinol, a commercial enzyme preparation from a *Penicillium*. It contained a double bond and phenolic groups. They concluded that the substance was a glucoside and described caffeic acid as a component of the aglycone. Shasha and Leibowitz (1961) prepared oleuropein with a melting point of 87-90°C and molecular weight corresponding to $C_{23}H_{30}O_{11}$. It reduced Fehling's and ammoniacal silver nitrate solutions and gave characteristic colour reactions for an aromatic nucleus, phenolic groups and sugar residues. Its concentration was found to be 0.6% (w/w) of the dry matter of the flesh of green fruit and up to 6% (w/w) of the cortex of the roots. It was found in the bitter green olives but not in black ones lacking this taste. They concluded that oleuropein is not a glucoside but a double ester of glucose and two acids: protocatechuic (3,4-dihydroxybenzoic) and an unsaturated oleuropeic acid. It occurred in green olives, as well as in leaves, stems and roots. They attributed to oleuropein the structure given in Figure 2.2. According to them oleuropein is hydrolyzed by acids and alkali and slowly attacked by extracts of tannase from *Aspergillus niger*. This extract was active against oleuropein at 35°C and pH 5. Oleuropein was not attacked by an α-glucosidase from baker's yeast, or by an active lipase in olive oil. The structure of oleuropein was given finally by Panizzi *et al.* (1960), it being

Fig. 2.2. a) Structure of oleuropein according to Shasha and Leibowitz (1961), b) Structure of oleuropein and its hydrolysis products as proposed by Panizzi *et al.* (1960), c) Chemical relation between oleuropein and some of its derivatives: 1: oleuropein, 2: dimethyloleuropein (Kuwajima *et al.* 1988)

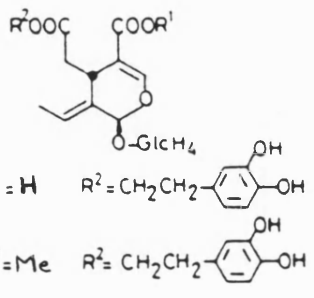
a



b



c



that of glucose, β-3,4-dihydroxyphenyl-ethanol and an acid which was identical to elenolic acid (Veer *et al.* 1957) (Fig. 2.2). They found also that the aglycone formed by β-glucosidase hydrolysis had a bitter taste. It is β-3,4 dihydroxyphenyl- ethanol connected through an ester linkage to an hexenoic acid.

Vazquez Roncero *et al.* (1961) prepared a pale yellow amorphous powder from extracts of green olives which they claimed to be at least in part oleuropein. They doubted its purity however claiming to have obtained several spots on a paper chromatogram. Cohen and Lifshitz (1969) and Cohen *et al.* (1967) also obtained a bitter substance from olives which had a strong absorbance around 340nm. They found also a good correlation between the bitterness of the olives and material having this absorbance. In recent years oleuropein has been isolated and its absolute structure determined (Walter *et al.* 1973; Inouye *et al.* 1970, 1974) through the application of High Performance Liquid Chromatography, Rotation Locular Countercurrent Chromatography and Droplet Countercurrent Chromatography (Federici & Bongi 1983; Vanhaelen *et al.* 1983; Kubo & Matsumoto 1984b; Terazawa 1986; Amiot *et al.* 1986; Willems 1988). Walter *et al.* (1973) listed the physical properties of oleuropein as well as its hydrolysis products (Table 2.6) and reported that oleuropein and its aglycone were bitter. Neither elenolic acid nor β-3,4-dihydroxyphenyl-ethanol was bitter. Recently Papadopoulos (pers.comm; Laboratory of Food Chemistry & Technology, Faculty of Chemistry, Aristotle University of Thessaloniki, Greece) obtained the HPLC analysis profile of commercial oleuropein both unhydrolysed and hydrolysed. Even the analysis of unhydrolysed commercial oleuropein gave more than one peak in the chromatogram indicating that either the commercial product is not pure or the oleuropein is a very unstable substance during storage.

Oleuropein occurs in variable amounts during the growth and maturation

Table 2.6. Physical properties of oleuropein and its hydrolysis products

Compound	Elemental composition	Molecular weight	UV absorption max (nm)	Mass spectral peaks	
				M+ Highest	MW fragments
Oleuropein	$C_{25}H_{32}O_{13}$	540.5	232 (e=14.135) 282 (e=2.973)	360 (0.7) 225 (0.6) 178 (1.4) 165 (3.5)	
β-3,4-Dihydroxy phenethyl alcohol	$C_8H_{10}O_3$	154.0626	220 (e=4.994) 283 (e=2.426)	154(19.7) 113 (2.4) 105 (2.0)	123(57.5)
Elenolic acid	$C_{11}H_{14}O_6$	242.0790	239 (e=10.729)	242 (0.8)	224 (1.7) 211 (1.1) 196 (9.0)
Aglycone	$C_{19}H_{22}O_5$	378.1321	225 (e=12.237) 285 (e=2.176)	378 (5.5)	346 (7.5) 243 (3.5) 211 (5.5)

Based on Walter *et al.* (1973)

of fruit and between different varieties of olives. A reduction in oleuropein content during maturation is accompanied by the accumulation of dimethyloleuropein and elenolic acid (Fig. 2.2; Amiot *et al.* 1986), and it is split naturally in the fruit by an esterase (Amiot *et al.* 1989) and β-glucosidase (Cruess & Alsberg 1934). Oleuropein has a common distribution in *Oleaceae*. It has been isolated with other secoiridoid glucosides, ligstroside and oleuroside, not only from *Olea europaea* (Kubo & Matsumoto 1984a; Kubo *et al.* 1985; Gariboldi *et al.* 1986; Kuwajima *et al.* 1988), but from *Olea capensis, Olea africana* (Tsukamoto *et al.* 1985), *Fraxinus mandshurica, Syringa vulgaris* (Terazawa 1986), *Ligustrum vulgare* (Willems 1988), *Fraxinus japonica* (Inouye *et al.* 1975a) and *Ligustrum japonicum* (Inouye *et al.* 1982). Oleuropein as well as other phenolic compounds may be utilised by some microorganisms (Garrido-Fernandez & Vaughn 1978; Balloni *et al.* 1977; Pelagatti 1981-1983).

Little attention has been given as yet to the contribution of oleuropein and other phenolic compounds to the bitter taste of virgin olive oil. Recently Gutierrez *et al.* (1989) correlated bitterness with the area of peaks coming out from a HPLC separation.

The following section describes the Materials and Methods and the advanced analytical techniques used to analyse phenolic extracts of olives and olive oil.

MATERIALS & METHODS

Olives

Sampling

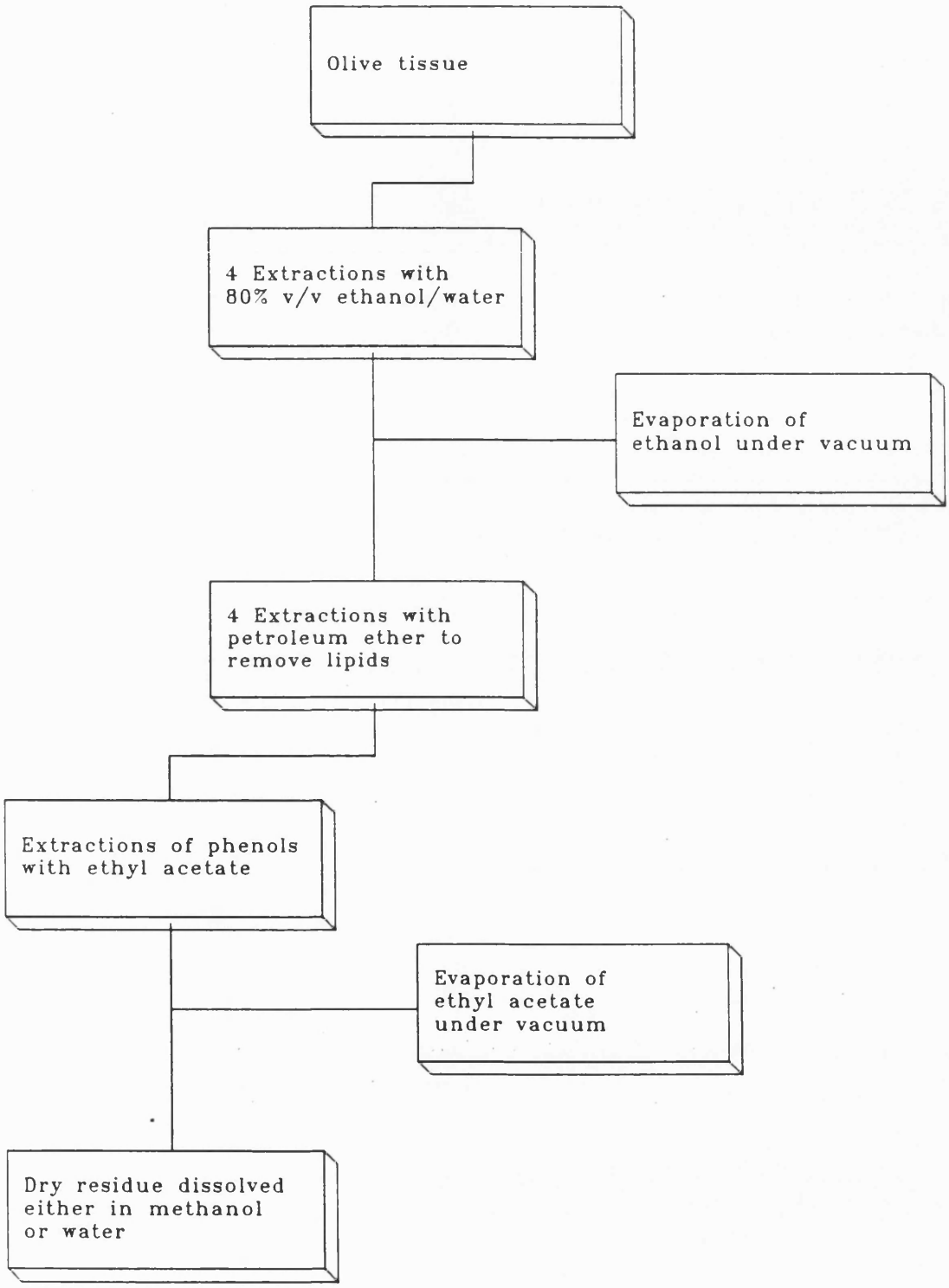
From October to December olive fruits were harvested in different areas of Greece for chemical analysis. Olives of different varieties and at different stages of maturation (green or black) were weighed (200g) and stored at 3°C for 2 days until analysis.

Extraction of phenolic compounds of olive fruits

Various extraction systems were explored and the most effective one (discussed below) was selected from the results of bio-assays using four strains of bacteria: *Pseudomonas fragi, Staphylococcus aureus, Bacillus cereus* and *Lactobacillus* sp. Paper discs (6.0mm, Whatman No 2017, 006) were soaked with olive extract obtained using different solvent every time - such as water, acetone, methanol, ethyl acetate, water/ethyl acetate (40:60v/v), water/methanol (40:60v/v)- and placed on the surface of Plate Count Agar in Petri dishes inoculated with the above microorganisms. After overnight incubation at 25°C the zones of inhibition were measured.

Olives (200g) - fresh and frozen in liquid nitrogen - after removal of the stones, were crushed in a mixer (Kenwood chef) (Fig. 2.3). The mixture was homogenized immediately in 80% (v/v) ethanol in water. After agitation at 4°C for 20 min followed by filtration (Whatman No.1), the residue was extracted again with 80% (v/v) ethanol. Aqueous-alcohol extracts were collected and evaporated under vacuum. Four successive petroleum ether extractions removed pigments and most of the lipids. The phenolic compounds were then extracted with ethyl acetate. After three succesive

Fig. 2.3. Flow chart for extraction of phenolic compounds from olive tissue



Additional details in text

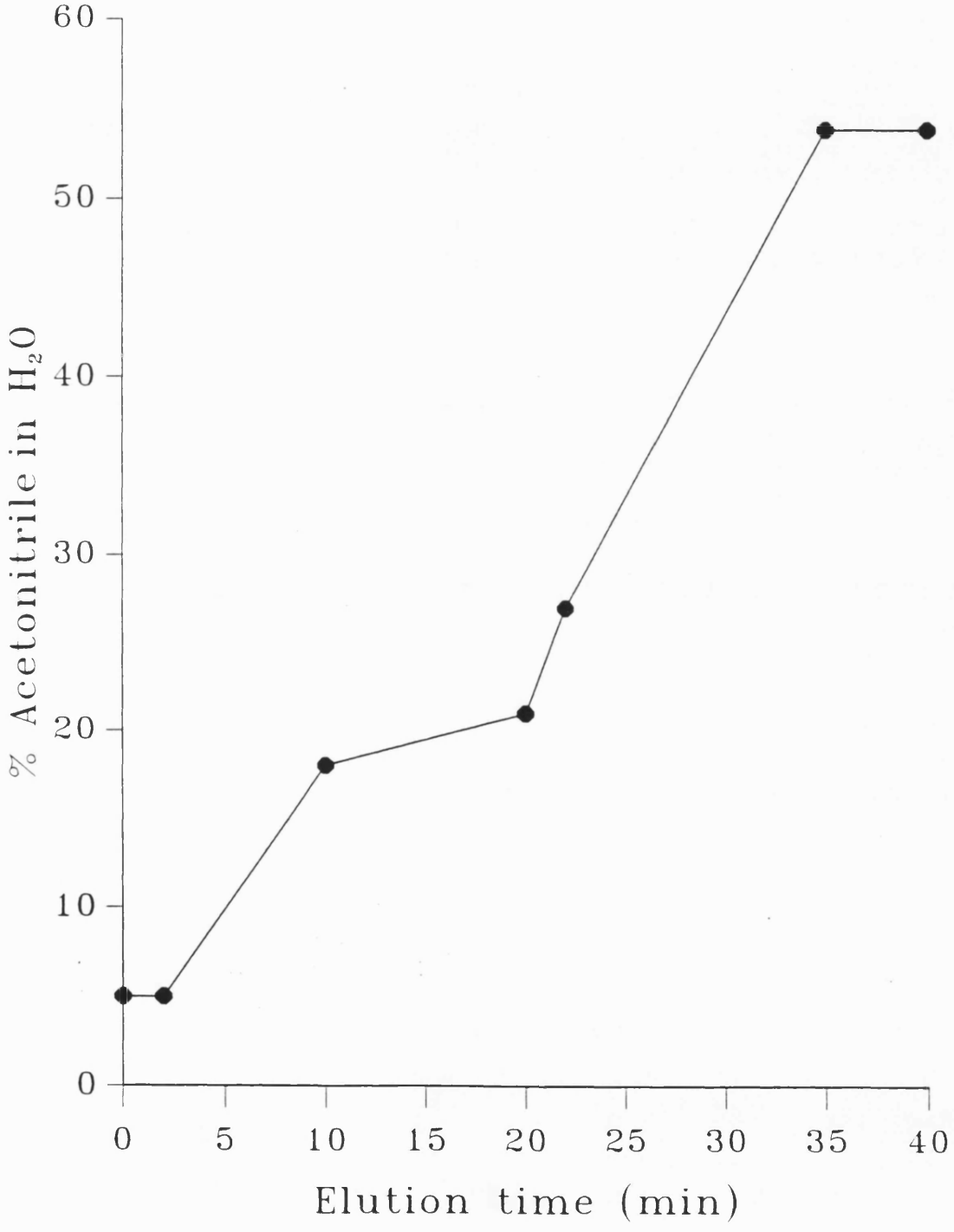
extractions, the ethyl acetate was removed under vacuum and 0.5g of the dry residue dissolved in 10ml water. Another part of the residue was dissolved in methanol and used for HPLC analysis. For comparative purposes olive samples were extracted with the method suggested from Amiot *et al.* (1986). Their method differs from the above in the addition of ammonium sulphate (20% w/v), orthophosphoric acid (2% w/v) and methanol to the ethyl acetate during the extraction.

HPLC Analysis

The chromatographic profile of the phenolic extract as well as the purity of oleuropein (Extrasynthese, 69730 Genay, France) were checked by HPLC. The analyses were carried out with an LCD Milton Roy high-pressure liquid chromatograph consisting of: two Model Constametric pumps, a Rheodyne 7125 injector, a Spectromonitor variable-wavelength detector set at 280nm, a Chromatograph Control Module microprocessor, and a Model S-201 GP printer. An FSA 25cm APEX C8 (particle size, 10μm) column was used for reverse-phase analysis and a solution of acetonitrile/H_2O, adjusted to pH 2.6 with orthophosphoric acid, was used as the mobile phase. The program was run isocratically with 5% (v/v) acetonitrile in water for the first 2min, the concentration being increased to 18% at 10min, 21% at 20min, 27% at 22min and 54% at 35min (Fig. 2.4). The solvents were HPLC grade. The flow rate was 4 ml/min and the injection volume 10μl.

Reference phenolic substances such as oleuropein (Extrasynthese, 69730 Genay, France), tyrosol, hydroxycinnamic acid, syringic acid, gallic acid, rutin, ferulic acid, 3,4-dihydroxy-phenylacetic acid, vanillic acid, protocatechuic acid, caffeic acid (Aldrich Chemical Co. Ltd) and veratric acid (Sigma Chemical Co.) were diluted in methanol and analysed with the

Fig. 2.4. Gradient solvent program used for the HPLC analysis of phenolic extracts from olive tissue and olive oil



same program (described above) by HPLC as standards or were added to a sample extract.

Olive oil

In order to test the effect of phenolic compounds on the taste of olive oil, different olive oils (Greek extra virgin, Italian extra virgin, blends of virgin olive oils) as well as sunflower oil were examined. Elais S.A. (the greatest olive oil company in Greece) provided us the Greek extra virgin olive oil while all the other oils were purchased from supermarkets.

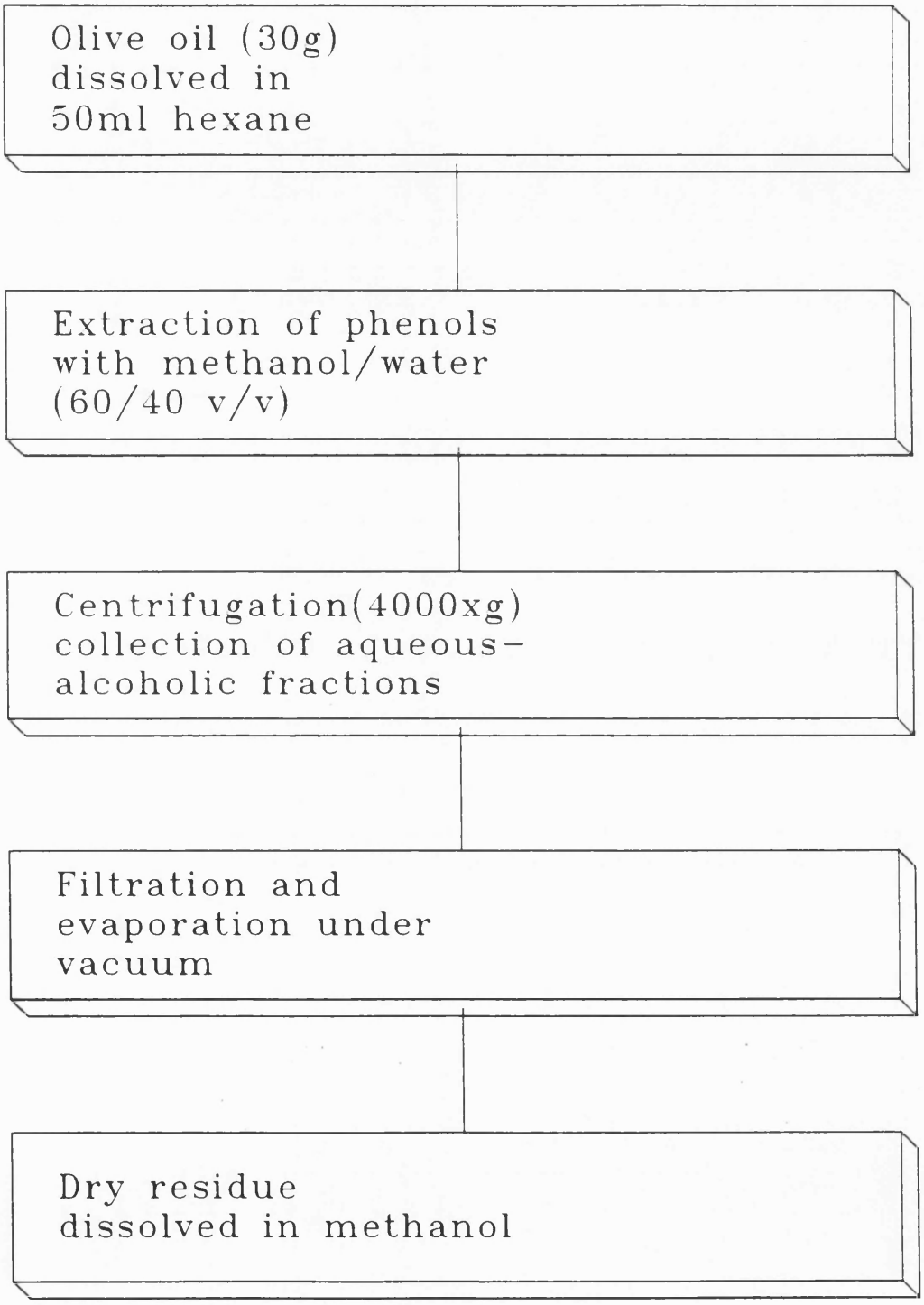
Extraction of phenolic compounds of oil

Oil (30.00 + 0.01 g) diluted in 50ml hexane was extracted with 3 x 50 ml of methanol: water (60: 40 v/v) - thorough mixing was achieved with a Kenwood chef mixer (Graciani Constante & Vazquez Roncero 1980). Two fractions were separated by centrifugation (300 x **g** for 10 min). The aqueous-alcoholic fractions from each extraction were filtered through damp Whatman No. 1 filter paper and evaporated under vacuum at 35-40°C (Fig. 2.5). The residue was dissolved in 2 ml methanol, filtered (Millipore 0.22μm) and used for HPLC as described above.

Determination of acidity

The method of Kiritsakis (1988) was used. An oil sample (28.2g) in an Erlenmeyer flask (250 ml) was mixed with 50 ml ethanol (95% pure) and 2 ml phenolphthalein (1% w/v in 95% ethanol) and titrated slowly with 1M NaOH until a pink colour appeared. The acidity was estimated thus: acidity (% oleic acid) = ml NaOH x Molarity NaOH x 0.282* x 100 x g^{-1} (*: 0.282 = mg of oleic acid).

Fig. 2.5. Flow chart for extraction of phenolic compounds from olive oil



Additional details in text

Organoleptic (sensory) evaluation

The oil samples were evaluated for bitterness by 7 members of a taste panel, four of whom were very familiar with olive oil flavour and taste and three were not. They were asked to taste different oils, with or without the addition of oleuropein, and to score bitterness. For this purpose a scale of 1 to 5 was used: 1 indicates imperceptible, 2 slight, 3 moderate, 4 great and 5 extreme bitterness.

RESULTS

Olive extract

In order to obtain an olive extract containing as much as possible of the phenolic compounds existing in olives and hence to possess the highest antimicrobial activity (see Chapter 3), different solvents (Table 2.7) were used. Table 2.7 shows that of the 6 solvents tested, ethyl acetate was the most efficient as judged by the response of the test organisms.

The typical profile of ethyl acetate extracts of olives analysed with HPLC is shown in Fig. 2.6. Some of the peaks on this chromatograph were identified with: protocatechuic acid (peak 3), dihydroxy-phenylacetic acid (peak 5), tyrosol (peak 6), vanillic acid (peak 7), rutin (peak 10), hydroxycinnamic acid (peak 12) and oleuropein (peak 13). Among the others, syringic acid could not be separated easily from caffeic acid (peak 8) and ferulic from veratric acid (peak 11). All these compounds had very similar retention times when run as reference samples under the conditions used in this part of my study (Table 2.8). The identification of the peaks was achieved using reference phenolic substances and/or adding a solution of the reference substance to a sample extract. In the first case a chromatogram of a single substance was obtained (Fig. 2.7a-f and Fig. 2.8a). In the second, the added substance increased the amount of the one existing in the extract and hence gave a higher peak in the chromatogram (Fig. 2.8b,c). The Retention Times (RT) of the compounds and their profiles used as standards are shown in Table 2.8 and in Figure 2.7a-f respectively.

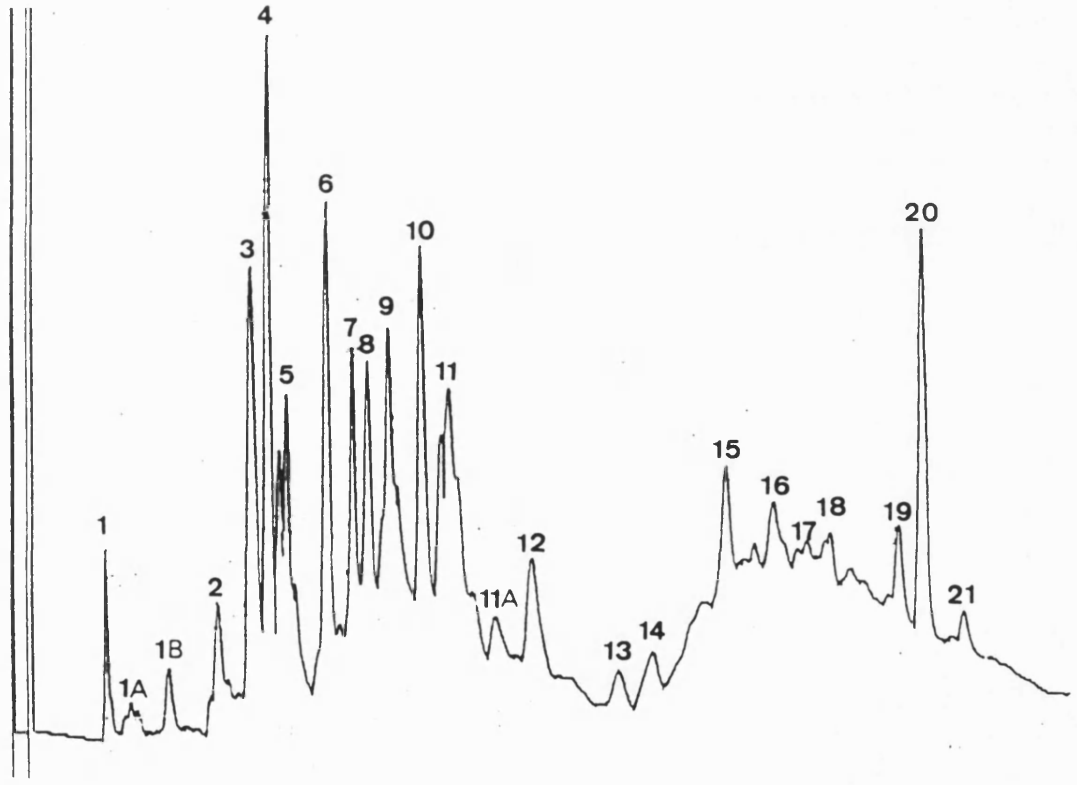
Oleuropein (peak 13) was among the most important substances identified (Fig. 2.9b). This peak was identified in many ways with oleuropein. First the RT obtained from the chromatogram (Fig. 2.8a) of the commercially pure oleuropein was compared with that of the peak of the green olive extract

Table 2.7. Inhibition zone (mm) caused by phenolic compounds of green olives extracted with different solvents, on various bacteria growing as a lawn on Plate Count Agar with incubation at 25°C

Organism	Solvent used for extraction#					
	water	acetone	methanol	ethyl acetate	water/ ethyl acetate	water/ methanol
Pseudomonas fragi	5	6	1	15	12	1
Staphylococcus aureus	6	6	12	2	18	11
Bacillus cereus	1	11	15	19	17	nd
Lactobacillus sp.*	8	8	9	13	nd	nd

nd: not determined
: the extraction was done as described in Materials and Methods
* : isolated from olives

Fig. 2.6. HPLC analysis of an ethyl acetate extract of black olives



Numbers of peaks are referred to in the text and in Table 2.8

Table 2.8. Retention times of the reference phenolic compounds detected by HPLC

Compound*	RT	Peak No.
Gallic acid	6.57	2
Protochatechuic acid	9.03-9.31	3
3,4-Dihydroxyphenylacetic acid	9.48-9.79	5
Tyrosol	10.84-11.17	6
Vanillic	12.39-12.69	7
Caffeic acid	12.53-12.84	8
Syringic acid	12.74-13.04	8
Rutin	14.75	10
Ferulic acid	16.39	11
Veratric acid	16.59	11
Hydroxycinnamic acid	19.60	12
Oleuropein	22.01	13

* : Oleuropein was purchased from Extrasynthese, 69730 Genay, France, veratric acid from Sigma Chemical Co. and the rest of the compounds from Aldrich Chemical Co. Ltd.

Fig. 2.7. HPLC analysis of reference phenolic compounds diluted in methanol: a) gallic acid, b) rutin, c) ferulic acid, d) vanillic acid, e) protocatechuic acid and f) caffeic acid

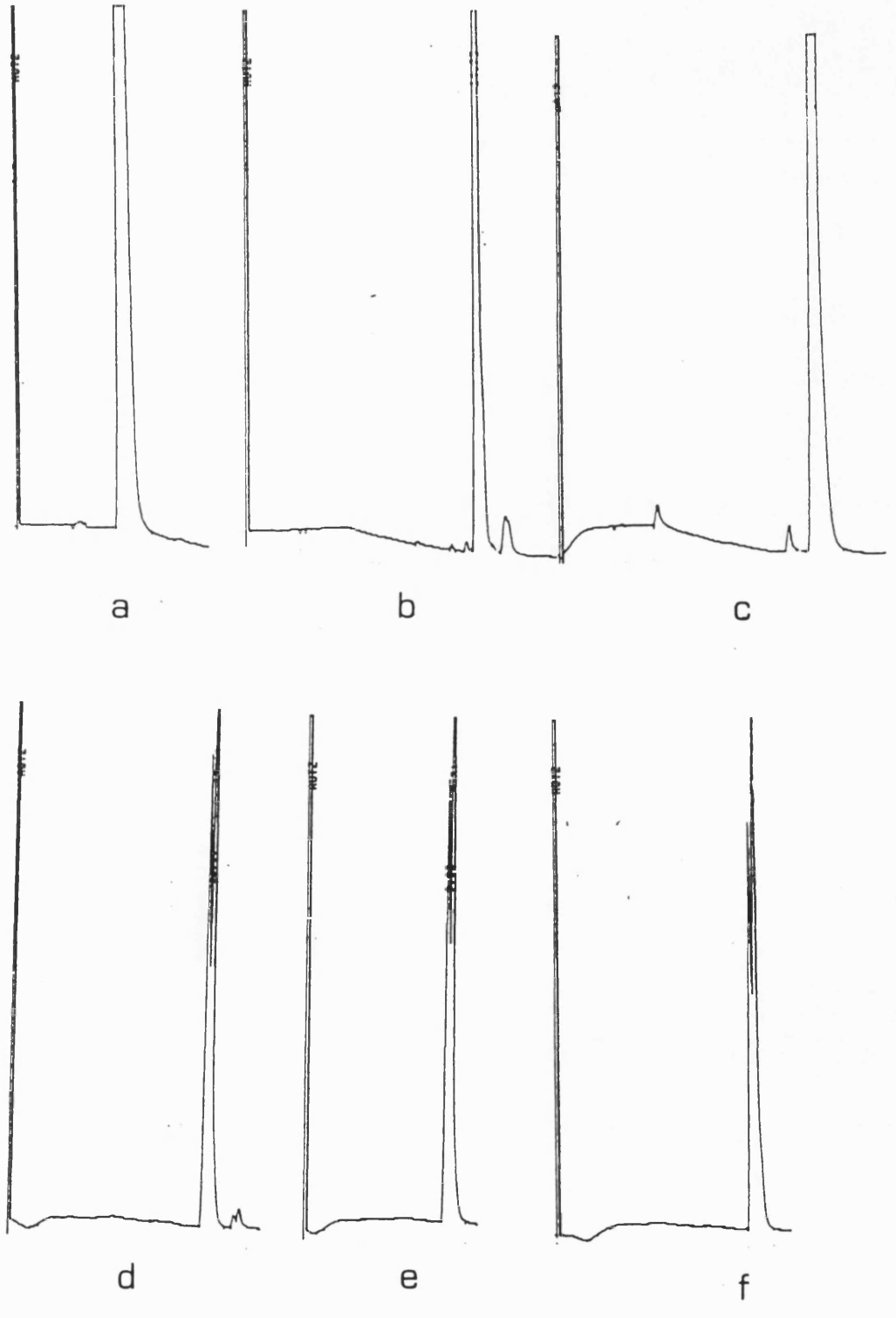
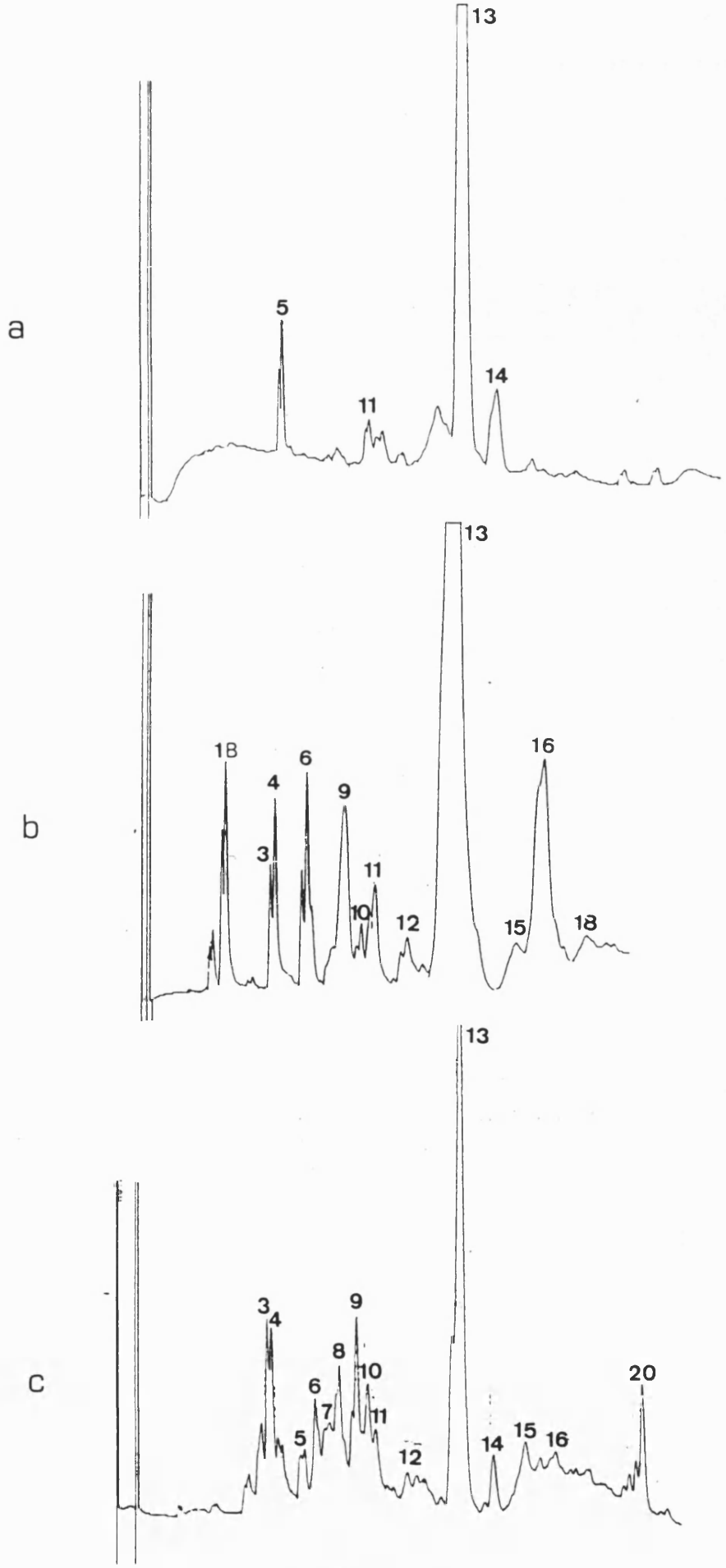
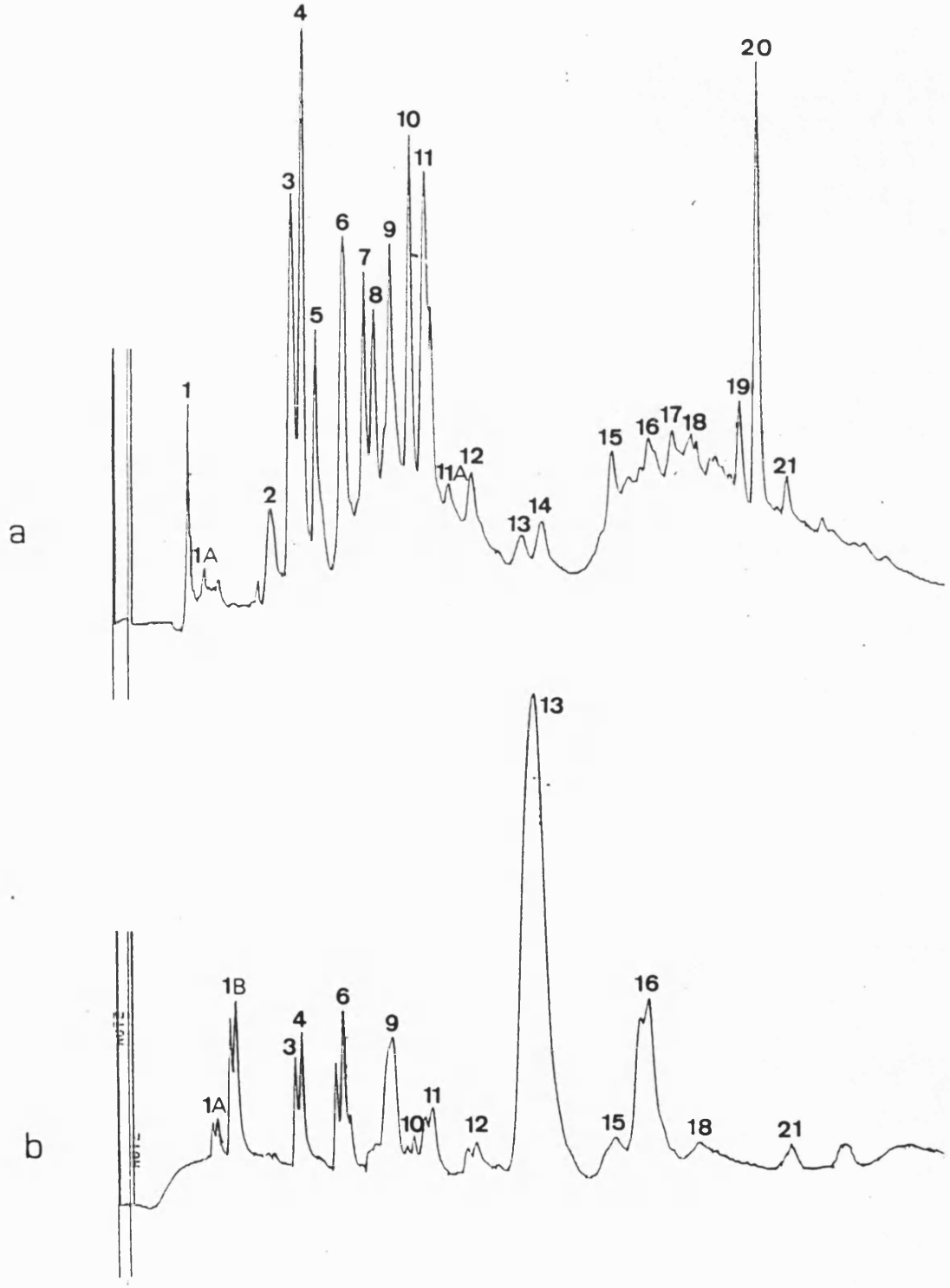


Fig. 2.8. HPLC analysis of a) commercially pure oleuropein (peak 13), b) pure oleuropein added to a phenolic extract of green olives and c) pure oleuropein added to a phenolic extract of black olives



For peak numbers, see Table 2.8.

Fig. 2.9. HPLC analysis of ethyl acetate extract of a) black olives and b) green olives



For peak numbers, see Table 2.8.

(Fig. 2.9b). It is obvious from this chromatogram of the pure substance (Fig. 2.8a) that oleuropein was not the sole peak, there being some other but smaller peaks also (dependant on the attenuation-sensitivity used). This indicates that purchased oleuropein was not completely pure, which is in agreement with the manufacturer's statement that their product is 80% pure. Similar results were obtained by Papadopoulos (pers.comm.; Laboratory of Food Chemistry & Technology, Faculty of Chemistry, Aristotle University of Thessaloniki, Greece). The oleuropein was identified in extracts of black (Fig. 2.8c) as well as of green olives (Fig.2.8b). In Fig. 2.9 two typical HPLC profiles of phenolic extracts are presented: Chromatograph A was obtained from a phenolic extract of black olives and chromatograph B of green ones. The differences between these two chromatograms are notable. The most dramatic difference is in that of the concentration of oleuropein (Fig. 2.9; peak 13). Indeed the two chromatograms in Fig. 2.9 show that the amounts of oleuropein compared to the other phenolic substances in the extract from green olives were considerably higher than in black ones.

It was highly probable that the peak (No. 4) having RT 9.33 in the chromatographs of Figures 2.6 & 2.9a,b was hydroxytyrosol. This peak was considerably smaller than that of oleuropein in green olives but was one of the major peaks in black olives from which oleuropein had almost disappeared. Further evidence that led me to suspect that this peak was hydroxytyrosol was the fact that two of its chemically related derivatives, dihydroxyphenylacetic acid and tyrosol, were identified with the peaks (5) and (6) respectively using standards, both of which absorb very close to hydroxytyrosol.

Olive oil

The acidity of a range of olive oils is given in Table 2.9. In Fig. 2.10a-c the chromatographic profile of two different Greek olive oils (2.10b-c) are

Table 2.9. Acidity and bitterness of various oils

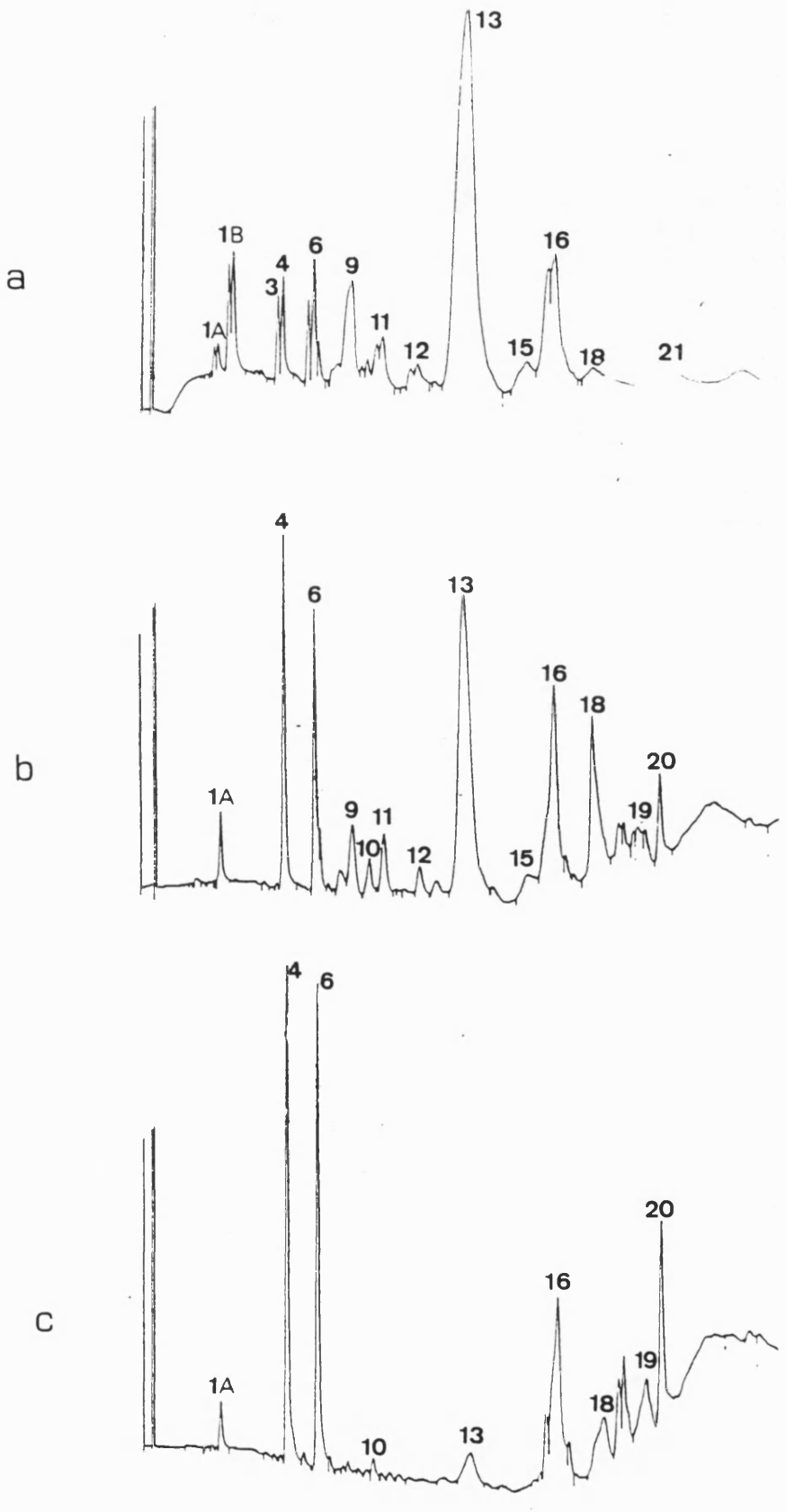
Oil[@] (Sample No)	Bitterness	Acidity[$]
Fresh virgin olive oil (No. 3)	5*	0.53
Fresh virgin olive oil (No. 6)	2	0.43
Fresh virgin olive oil (No. 7)	1.5	0.63
Fresh virgin olive oil (No. 8)	2.5	0.60
Fresh virgin olive oil (No. 208)	4	0.53
Sunflower oil	1	
Sunflower + oleuropein	1.5	
Italian olive oil	3.7	0.60
Italian olive oil + oleuropein	4.2	
EEC Blended oil	2	0.54
EEC Blended oil + oleuropein	3.1	

@ : All the fresh virgin olive oils were provided by Elais S.A. and the rest were purchased from a supermarket

* : (1) imperceptible, (2) slight, (3) moderate, (4) great and (5) extreme bitterness

$: % oleic acid

Fig. 2.10. HPLC analysis of phenolic extract of a) green olives, b and c) two fresh virgin olive oils, samples No 3 and No 8 (Table 2.9) respectively



For peak numbers, see Table 2.8

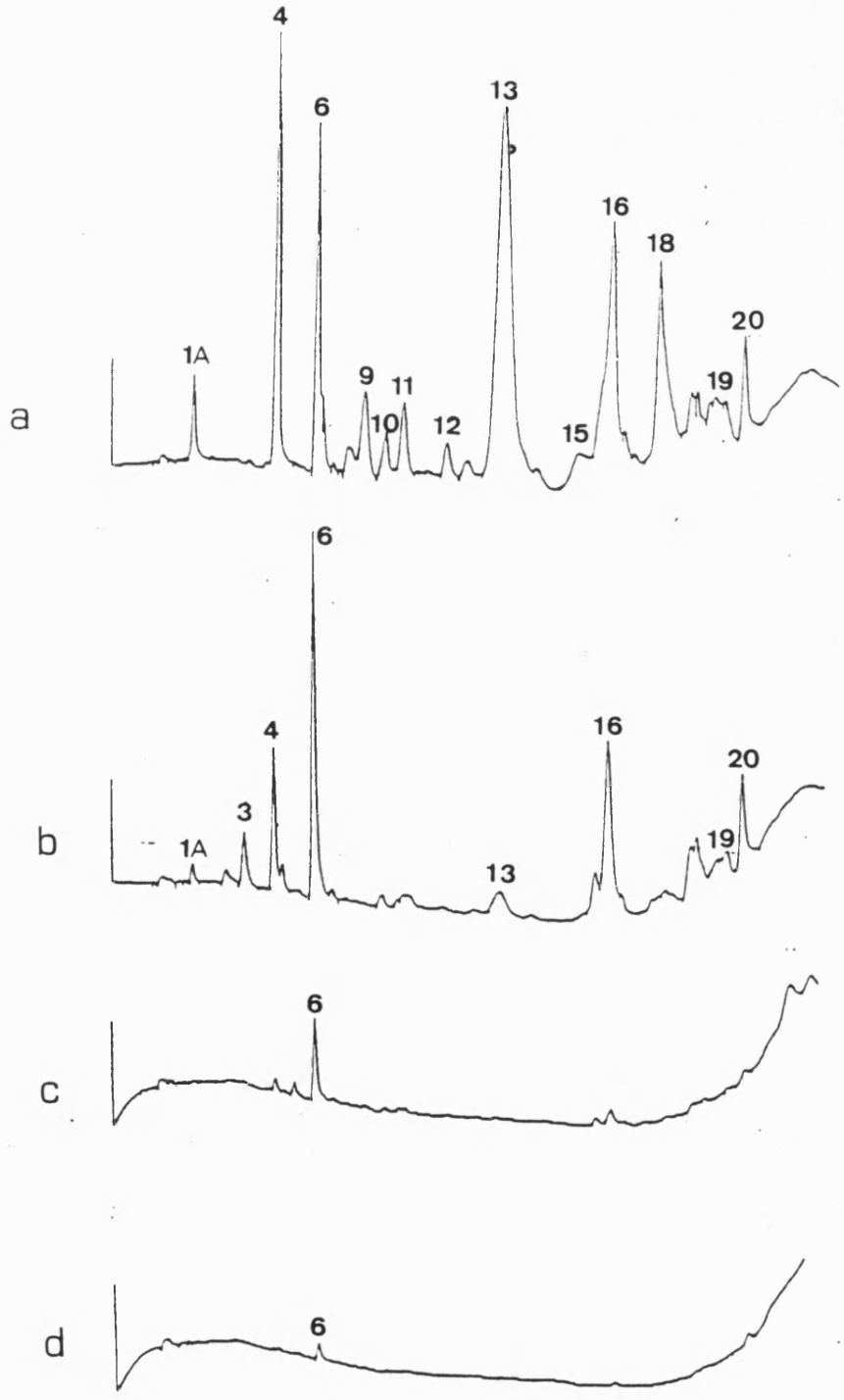
presented in comparison with the profile of the green olive extract (Fig. 2.10a). A few peaks only have been identified with those described above. It is evident that the principal peaks are No 4 & 6. These peaks have been identified with hydroxytyrosol and tyrosol respectively.

Figure 2.11a-d shows the phenolic profile of different olive and vegetable oils. It is evident that the amount and type of phenolics differ significantly among these. In particular the most complex phenolic profile (Fig. 2.11a) was found in fresh extra virgin olive oil. The second most complex (Fig. 2.11b) was an extra virgin olive oil which had been stored on a super market shelf. The third (Fig. 2.11c) was a mix (blended) olive oil from different EEC olive oil producing countries stored in a super market. The fourth oil, a vegetable oil (sunflower), had no obvious phenolic peaks.

The effect of storage on the concentration of phenolics is evident in Figures 2.12a-b, 2.13a-b and 2.14a,b. It is clear that there was a reduction of peak No.4, 16 and 18 in stored products. The effect of storage on extracted samples in methanol is shown in Figure 2.15a-d. Indeed it is clear that the chromatographic profile of the phenolics changed dramatically, the most dramatic being associated with peaks No.4 and 13.

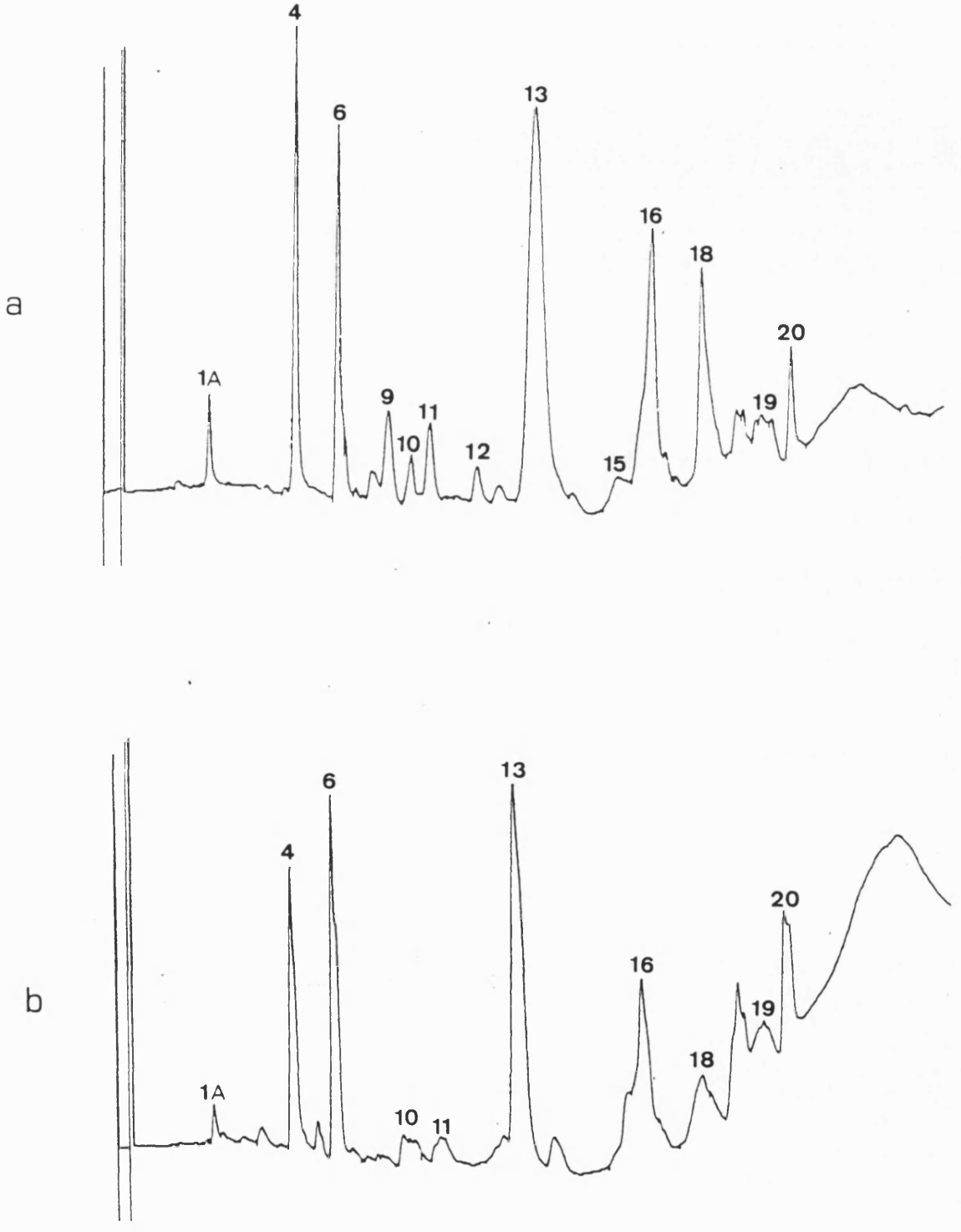
The intensities of bitterness of six extra virgin olive oils, one blended olive oil and a vegetable (sunflower) oil, are shown in Table 2.10. It was found that the addition of oleuropein increased the bitterness of oils.

Fig. 2.11. HPLC analysis of phenolic extract of a) fresh extra virgin olive oil, b) stored extra virgin olive oil, c) blended olive oil of EEC countries and d) sunflower oil



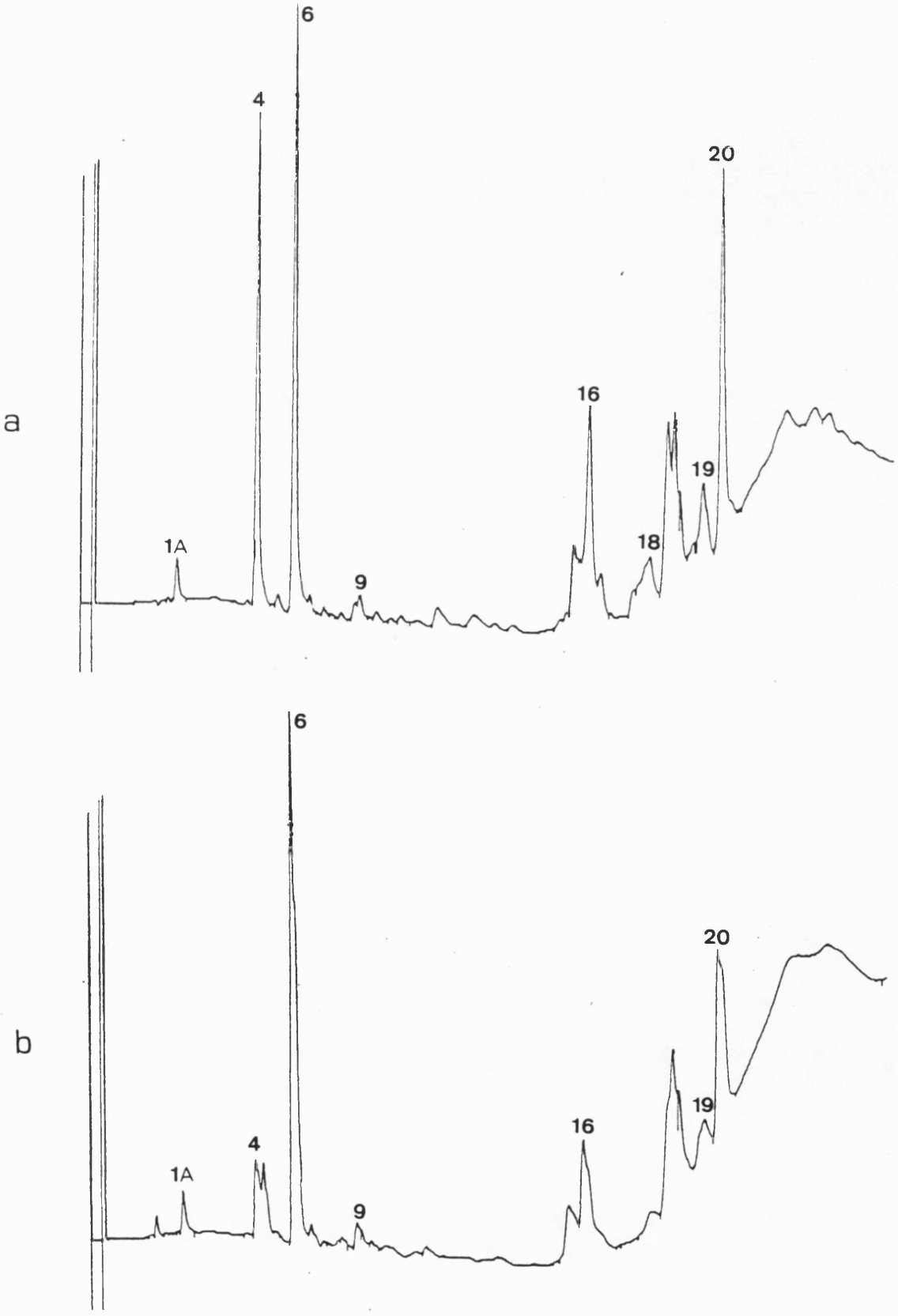
For peak numbers, see Table 2.8

Fig. 2.12. HPLC analysis of phenolic extract of a) fresh extra virgin olive oil, sample No 3 and b) the same olive oil after 2 months storage in dark at room temperature



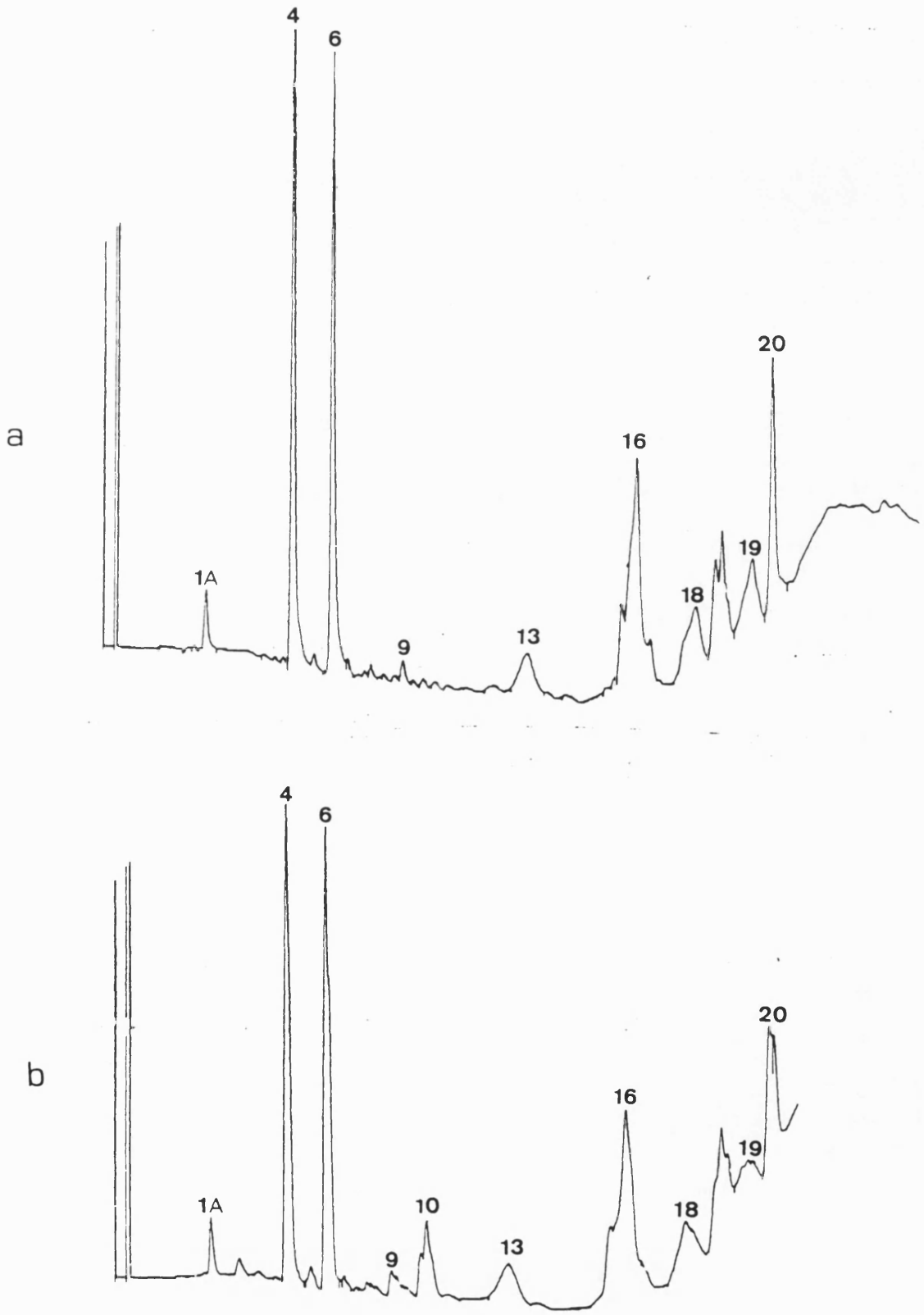
For peak numbers, see Table 2.8

Fig. 2.13. HPLC analysis of phenolic extract of a) fresh extra virgin olive oil sample No 7 and b) the same olive oil after 2 months storage in dark at room temperature



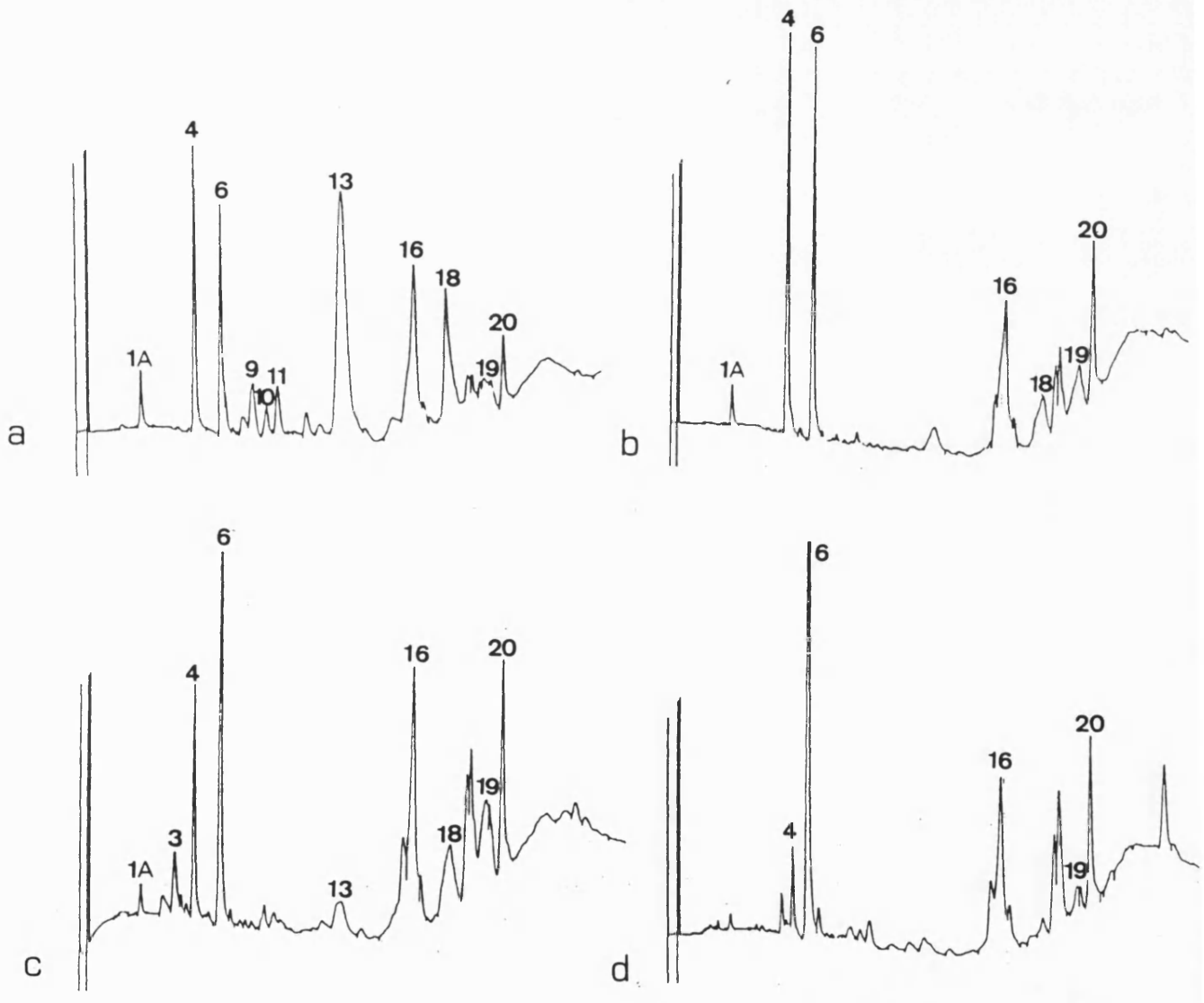
For peak numbers, see Table 2.8

Fig. 2.14. HPLC analysis of phenolic extract of a) fresh extra virgin olive oil, sample No 8 and b) the same olive oil after 2 months storage in dark at room temperature



For peak numbers, see Table 2.8

Fig. 2.15. HPLC analysis of phenolic extract of a, b) fresh extra virgin olive oil, samples No 3 and No 8 respectively and c, d) the same extracts after 1 month storage in dark at room temperature



For peak numbers, see Table 2.8

Table 2.10. Samples of different oils and flavour score (bitterness) as scored by 7 judges

Oil (Sample No)	Judges 1	2	3	4	5	6	7	Average
Fresh virgin olive oil (No. 3)	5*	5	5	5	5	5	5	5
Fresh virgin olive oil (No. 6)	2	2.5	1.5	2	2	1	3	2
Fresh virgin olive oil (No. 7)	1	1	2	1	2	1.5	2	1.5
Fresh virgin olive oil (No. 8)	2	3	2	2	3	2.5	3	2.5
Fresh virgin olive oil (No. 208)	4	5	4	4	4	3	4	4
Sunflower oil	1	1	1	1	1	1	1	1
Sunflower oil + oleuropein	2	1	2	1.5	1	1.5	1.5	1.5
Italian olive oil	3	4	4.5	4	3.5	3.5	3.5	3.7
Italian olive oil + oleuropein	4	4.5	4.5	4.5	4	4	4	4.2
EEC Blended oil	2	1.5	1.5	2	2	2.5	2.5	2
EEC Blended oil + oleuropein	3	3	2.5	2.5	3	3.5	3.2	3.1

* : (1) imperceptible, (2) slight, (3) moderate, (4) great and (5) extreme bitterness

DISCUSSION

It is well known that phenolics and their derivatives are significant contributors to taste and odour of many plant products (Hermann 1972; Torres *et al.* 1987; Bailey *et al.* 1990) especially olives (Cruess & Alsberg 1934) and products thereof (Vazquez Roncero 1978, Drusas *et al.* 1988). Thus it is important to identify those that occur in olive fruits because of their contribution to the organoleptic attributes of olives and olive products and because of their potential use as natural antimicrobial agents or antioxidants. It was evident in this study (Fig. 2.6) that simple as well as complex phenol structures were present in olives. As the oil is processed, these are distributed between the product's organic and aqueous phases. Kiritsakis (1982) reported an average phenol value of 120ppm for Greek olive oil extracted by centrifugation. As demostrated by Kiritsakis and Markakis (1987), phenolic substances (eg. tyrosol, hydroxytyrosol) contribute to the stability of virgin oil to oxidation. Indeed Gutierrez *et al.* (1989) have established a good correlation of the aqueous-alcohol extract contents versus odour and flavour as determined by organoleptic tests.

I had difficulties in identifying some of the phenolic compounds (eg hydroxytyrosol) of most importance in the olive industry especially in olive oil technology (Kiritsakis 1982; Kiritsakis & Markakis 1987). This compound, which protects olive oil from oxidation, is not available commercially and has to be prepared in the laboratory from 3,4 dihydroxyphenylacetic acid (Papadopoulos & Boskou 1991). Some of the other phenolics listed in Table 2.4 were not available commercially - this proved to be a problem when seeking reference samples. In particular it was difficult to obtain verbascoside and dimethyl-oleuropein in order to identify these substances in our samples and to confirm or otherwise the observations of Amiot *et al.* (1986, 1989) that the above substances increase in amount

concurrently with the reduction of oleuropein.

The other phenolic substances found in substantial levels in my extracts of black olives and identified either substantively or tentatively with protocatechuic acid, tyrosol, hydroxytyrosol, caffeic acid, rutin, vanillic acid and ferulic acid have been identified previously in extracts of olive pulp, olive leaves and waste water after olive oil extraction (Ragazzi & Veronese 1967; Vazquez Roncero *et al.* 1974; Rodriguez *et al.* 1988).

Several authors have emphasized not only the importance of the extraction methods but also the instruments used (GC, HPLC) or other analytical systems (wavelenth used in UV determination of phenols, time of analysis after extraction) in determining the types of compounds obtained from various plants (Chao & Suatoni 1982; Torres *et al.* 1987; Bailey *et al.* 1990) and olives (Amiot *et al.* 1986; Tsukamoto *et al.* 1985). In this study an ethyl acetate extract was used for HPLC analysis as it was found to be the most active against bacteria (Table 2.7). Similar results have been reported by Rodriguez *et al.* (1988). It was evident in this part of my study also that there was poor stability of the phenolics either in samples or in raw material which had been stored for any period of time (Figs. 2.10, 2.11 and 2.15a-d). Recently Brenes Balbuena *et al.* (1992) found that there was a different chromatographic profile of phenolics from olives obtained when the extraction was done from fresh olives compared to the profile from olives treated with lye.

In this part of my study also a variety of phenolic compounds were identified and found to be present in olives and virgin olive oil. The phenolic profile, as analysed by HPLC, of green and black olives showed that the former contained almost the same range of phenolic compounds as black ones. An important phenolic compound - oleuropein - was present in high

amounts in green olives but not in black ones.

Total phenolic content can vary in different parts of the olive tree (Gariboldi *et al.* 1986; Kuwajima *et al.* 1988; Tsukamoto *et al.* 1985), in the same species at the different times of the year (Amiot *et al.* 1986; 1989) and in the same tissue at different stages of growth. In the present study, I have demonstrated such differences in olive fruits at different stages of growth (Figs. 2.9a,b)

The observation in my study that oleuropein occurs in large amounts in green olives but in very low amounts in black ones is in agreement with the findings of others (Cruess & Alsberg 1934; Shulman & Lavee 1976; Amiot *et al.* 1986, 1989). Amiot *et al.* (1986) noted that the oleuropein content increased rapidly at the beginning of the growth of the fruit. A very rapid reduction was first observed during the phase characterizing maturation of olives and then much slower reduction occurred after a change in the colour of the fruit. When olives were picked before full maturity (green), oleuropein made up a considerable amount (3-6% w/w) of the dry matter.

According to Barz and Koster (1981) the reduction in oleuropein content during fruit maturation was accompanied by the accumulation of two derivatives of oleuropein, dimethyloleuropein and elenolic acid glucoside (Fig. 2.2). This was confirmed by Amiot *et al.* (1989). However none of the derivatives of oleuropein such as 3,4-dihydroxyphenyl-ethanol (hydroxytyrosol), elenolic acid, oleuropein aglycone etc. noted *in vitro* have been reported as being natural constituents during the physiological development of olive fruit. However two glucosides of 3,4-dihydroxy-phenylethanol, the 4-monoglucoside and 4-diglucoside, were present in olive pulp (Vazquez-Roncero *et al.* 1974). Free tyrosol (hydroxyphenylethanol) and hydroxytyrosol (3,4-dihydroxyphenylethanol) were found mainly as constituents of waste water from olive oil mills (Ragazzi & Veronese 1967). In the present study tyrosol and hydroxytyrosol were present in most of the

fresh olive extracts while oleuropein was found only in two different extracts of green olives.

The considerable reduction of oleuropein content shows clearly that it is not an inert metabolic product but is re-utilized during the maturation phase. This phenomenon has often been stressed for other phenolic compounds in various plant organs (Strack *et al.* 1978; Linscheid *et al.* 1980), and it should be related to the active turnover of certain phenolic metabolites (Molderez *et al.* 1978; Barz & Koster 1981). The fact that dimethyl-oleuropein and elenolic acid accumulate in the olive fruit concurrently with the reduction of oleuropein suggests that these events may be related biochemically. There is the possibility that these two compounds are formed from oleuropein by the action of esterases. Esterase activity increased considerably during the first phase of maturation and reached a maximum during the "black" maturation phase (Amiot *et al.* 1989). The fruit of *Olea europaea* appears to accumulate only glucosylated derivatives of oleuropein (Vazquez-Roncero *et al.* 1974). These are probably less toxic than the aglycones to microorganisms. It is probable also that oleuropein may be converted by the action of glucosidases (Gariboldi *et al.* 1986) but the intermediate compounds are probably re-metabolised immediately in the fruit. It is also highly possible that the microbiological decomposition of oleuropein may occur in olives awaiting processing. The yeast, *Candida veronae,* has been used successfully to split oleuropein (Balloni *et al.* 1977; Pelagatti 1981-1983).

The importance of phenolic compounds in olive oil quality is well known, especially their correlation with the peroxide number, free fatty acidity, and sensory tests (Gutierrez *et al.* 1989; Montedoro *et al.* 1992a,b). These authors found oleuropein, coumaric, gallic, caffeic, syringic, ferulic and cinnamic acids in samples of virgin olive oil. With the exception of oleuropein similar results were obtained with my samples in respect of all of

the above mentioned phenolic acids. In my samples oleuropein was present also in high amounts in one of the fresh extra virgin olive oils, but not in the others. In contrast other phenolic compounds were found in all the virgin olive oils analysed but not in the blended and sunflower oil. All the phenolic compounds detected in olive oils were unstable during storage of either the olive oil or an extract therof.

CHAPTER 3

ANTIMICROBIAL PROPERTIES OF PHENOLIC OLIVE EXTRACTS AND OLEUROPEIN

Introduction

The occurrence of an inhibitor in green olives active against lactic acid bacteria was first noted by Vaughn (1954). Fleming and Etchells (1967) obtained an ethyl-alcohol extract from green olives and tested its inhibitory effect on the growth rates of lactic acid bacteria associated with the fermentation of olives. *Leuconostoc mesenteroides* was the most and *Lactobacillus plantarum* the least sensitive. *Pediococcus cerevisiae* and *Lactobacillus brevis* were intermediate in sensitivity. The degree of inhibition was correlated with the total phenol content of the extract. The levels of the inhibitor in the extract from olives was related inversely to the rates of fermentation. Sodium chloride was found to act synergisticaly with the inhibitor. The inhibitor was stable at 100°C but unstable at pH 10. Its effectiveness was increased by freezing olives. Freezing probably caused a release of the inhibitor due to physical changes in the olive tissue or it resulted in a chemical alteration. Cruess and Alsberg (1934) contended that the olive contains an emulsin-like enzyme that was liberated by freezing and possibly another enzyme that split or oxidised the bitter ester remaining after hydrolysis of the glucoside.

Juven *et al.* (1968a) confirmed that the increased rates of lactic fermentation of green olives which had been exposed to a hot alkali treatment stemmed in part from the destruction of this anti-lactic acid bacteria factor. The same investigators (1968b) demonstrated the antibacterial activity of an ethanol extract of green olives on *Lactobacillus plantarum* and related this to

phenolic compounds. They noted also that oleuropein, isolated according to the method of Panizzi *et al.* (1960), inhibited *Lactobacillus plantarum, Leuconostoc mesenteroides, Pseudomonas fluorescens, Micrococcus albus* and *Bacillus subtilis*. It did not inhibit *Aerobacter aerogenes, Escherichia coli, Candida albicans* and *Saccharomyces cerevisiae*.

Inhibitory effect on spoilage organisms

Fleming *et al.* (1969) noted that oleuropein was one of the six major phenolic compounds in an ethyl-acetate extract of olives. The major inhibitory substance, however, was a phenolic compound devoid of an acid-hydrolyzable reducing sugar, probably the aglycone of oleuropein. This bitter compound was much more inhibitory to lactic acid bacteria than oleuropein. They concluded in another study (Fleming *et al.* 1973) that green olives had an enzyme system which caused the hydrolysis of oleuropein to the antibacterial aglycone when olives were brined. On degradation the aglycone yields elenolic acid, a compound which is also antibacterial. Oleuropein but not the aglycone was present in brines of heated olives.

Later work offers another explanation for the brief, alkali pre-brining treatment minimizing the inhibition of the fermentation. According to Juven and Henis (1970), when untreated olives are brined, fermentation substrates, growth factors and phenolic antimicrobial compounds are released from the fruit. Following a brief alkali pre-brining treatment, the main cell constituents of the olive (sugars, B complex vitamins and amino acids - see Chapt.2) are released at a rate faster than that observed with untreated ones. The concentration of phenolic compounds in the brine also increased rapidly, but their inhibitory activities towards lactic acid bacteria were counteracted by the higher concentration of nutrients. As a result of the increased permeability of the heat-damaged cytoplasmic membranes, salt concentration

in the brines of the pretreated olives diminished to a level lower than that found in those of untreated olives. This also minimized the inhibitory effect. They confirmed also that the aglycone was more inhibitory than oleuropein on *Lact.plantarum*. Oleuropein at a level of 0.2% (w/v) inhibited *Geotrichum candidum*, but not *Saccharomyces cerevisiae, S.oviformis, S.carlsbergensis, Candida albicans, C. tropicalis, C.krusei* and *Pichia membranefaciens*. Among the fungi tested, *Trichoderma lignorum, Rhizopus* sp., *Rhizoctonia solani, Aspergillus niger, Phoma* sp. and *Penicillium cyclopium*, only the growth of a *Rhizopus* sp. and *Rhizoctonia solani* was partially arrested by 0.2% (w/v) oleuropein.

Fleming *et al.* (1973), who tested the inhibitory activity of an extract of frozen olives, pure oleuropein and its hydrolysis products, showed that two products, aglycone and elenolic acid, inhibited growth of lactic acid bacteria. The glucoside had no effect (Tables 3.1 & 3.2). Garrido Fernandez and Vaughn (1978) tested the effect of 0.2% and 0.4% (w/v) oleuropein in combination with 1% (w/v) glucose on microorganisms (Table 3.3). All of the cultures of bacteria and yeasts grew in the presence of 0.2 and 0.4% (w/v) oleuropein with or without glucose. Even so there was a difference in the time required for the development of turbidity in the presence of oleuropein. The difference was least with *Bacillus* spp. The cultures of *Rhodotorula* spp. did not exhibit any response. None of the species of lactic acid bacteria grew with oleuropein as the sole carbon source. There was a variation, however, in the time required for development of strong visible turbidity in the media containing 0.2% (w/v) oleuropein as compared with those containing 0.4% (w/v) of that compound. There was an inhibitory effect on lactic acid bacteria but not all cultures were affected to the same extent by an increased concentration of oleuropein. They concluded that the bitter glucoside is utilized by most, if not all, of the microorganisms associated with the fermentation of olives. The inhibitory activity of aglycone

Table 3.1. Inhibition of lactic acid bacteria by hydrolysis products of oleuropein as indicated by paper disc bioassay

Bacteria	Oleuropein	Aglycone	Elenolic acid	Dihydroxy-phenyl-ethanol	Methyl o-methyl elenolate
Lactobacillus plantarum	-	+	+	-	-
Pediococcus cerevisiae	-	+	+	-	-
Lactobacillus brevis	-	+	+	-	-
Leuconostoc mesenteroides	-	+	+	-	-

+ : inhibition
- : no inhibition
Based on Fleming *et al.* (1973)

Table 3.2. Antimicrobial spectrum of extract from olives by paper disc bioassay

Microorganism	Extract containing oleuropein	Acid-hydrolysate of oleuropein	Extract from frozen olives
Lactobacillus			
plantarum W50	-	+	+
brevis 50	-	+	+
Pediococcus cerevisiae 39	-	+	+
Leuconostoc mesenteroides 42	-	+	+
Staphylococcus aureus	+	+	+
Bacillus subtilis	+	+	+
Enterobacter			
aerogenes	-	-	-
cloacae NRRL B-414	-	-	-
Escherichia coli	-	-	+
Salmonella typhimurium	-	+	+
Pseudomonas			
fluorescens	-	-	+
solanacearum	+	+	+
lachrymans	-	+	+
Erwinia			
carotovora	-	+	+
tracheiphila	-	-	+
Xanthomonas vesicatoria	-	+	+
Corynebacterium michiganese	-	-	+
Saccharomyces			
rosei	-	-	-
cerevisiae var. *ellipsoideus*	-	-	-
Hansenula subpelliculosa	-	-	-
Kloeckera apiculata	-	-	-
Debaryomyces membranefaciens	-	-	-
Pichia membranefaciens	-	-	-
Candida krusei	-	-	-

+ : inhibition
- : no inhibition
Based on Fleming *et al.* (1973)

Table 3.3. Microorganisms tested for the effectiveness of the antimicrobial action of oleuropein

Bacteria	
Gram positive	Gram negative
Bacillus	*Achromobacter* spp.
macerans	*Aerobacter*
polymyxa	*aerogenes*
pumilus	*cloacae*
Lactobacillus	*Aeromonas*
bulgaricus	*liquefaciens*
casei	*Alcaligenes*
delbrueckii	*faecalis*
fermenti	*Cellulomonas*
lactis	*flavigena*
plantarum	*Escherichia*
Leuconostoc	*intermedia*
mesenteroides	*Paracolobactrum*
citrovorum	*aerogenoides*
dextranicum	*Xanthomonas* spp.
Micrococcus spp.	*stewartii*
Streptococcus	
cremoris	
diacetilactis	
faecalis	
lactis	
liquefaciens	
thermophilus	
Kurthia	
bessonii	

Yeasts
Rhodotorula
glutinis var. *glutinis*
minuta var. *minuta*
rubra

Based on Garrido-Fernandez & Vaughn (1978)

and elenolic acid fractionated by HPLC on *Lact.plantarum* was shown also by Federici and Bongi (1983). As the aglycone inhibited *S.cerevisiae* and *B.subtilis,* Kubo and Matsumoto (1985) concluded that this is the active moiety of oleuropein.

Recently Ruiz Barba *et al.* (1993) confirmed that three glucosidic compounds, verbascoside, oleuropein and luteolin-7-glucoside, are present in untreated olive brines but not in the NaOH-treated ones. Hydroxytyrosol, tyrosol and vanillic acid were found in both brines. All the above phenolic compounds, except hydroxytyrosol, showed no bactericidal effect against *Lact.plantarum* ATCC 8014 when assayed as single fractions in the concentrations found in brines. They exhibited however a remarkable additive antimicrobial effect when tested together two-by-two. Hydroxytyrosol was highly bactericidal when tested alone or in combination with oleuropein or verbascoside.

Inhibitory effect on pathogens

Oleuropein stimulated the growth of *Aspergillus parasiticus* and *Penicillium* spp. but inhibited the production of aflatoxin (Gourama & Bullerman 1987; Gourama *et al.* 1989). Phenolic extracts of olive callus tissues, containing mainly caffeic acids and to a lesser extent catechin and coumarins, inhibited aflatoxin production by 90% without inhibiting the growth of *Aspergillus flavus* on media containing 0.5 or 1.0% (w/v) of extract (Paster *et al.* 1988). Phenolic substances in waste waters (alpechin) from olive oil mills exhibited antimicrobial activity on *Bacillus* spp. in soil and inhibited sporulation and germination of *Bacillus megaterium* in the laboratory (Rodriguez *et al.* 1988; Paredes *et al.* 1986, 1987; Moreno *et al.* 1987; Gonzalez *et al.* 1990; Perez *et al.* 1992). These substances act as growth inhibitors in the wastes therby delaying its biodegradation.

Microorganisms resistant to the antimicrobial effect of phenolic substances

are obviously very important in the degradation of wastes from olive oil factories. Organisms such as *Saccharomyces* sp., *Torulopsis* sp. (Mas & Peinado 1984), *Torulopsis* sp. MK-1, *Saccharomyces norbensis* MC-1, *S.oleaceous* MC-2 and *S.oleaginosus* have been shown to grow well and ferment the sugars of waste waters (Bambalov *et al.* 1989).

Mode of action

Fogg and Lodge (1945) first proposed that the mechanism of antimicrobial activity of phenols was the inactivation of cellular enzymes. Vas (1953), however, proposed that phenol attacked the cytoplasmic membrane releasing intracellular constituents. Judis (1963) found that up to 50% of the Na glutamate-3,4-^{14}C and 12% of the $NaH_2{}^{32}PO_4$ were lost by *E.coli* in the presence of the phenolics. It was concluded that the loss was due to a weakening or destruction of the permeability barrier of the cell membrane.

In 1972 Juven *et al.*, studying the mode of action of oleuropein, concluded that it is a surface active agent affecting the cell membranes. It caused haemolysis of human erythrocytes and induced significant leakage of glutamate, potassium, and inorganic phosphate from *Lact.plantarum.* It had no effect on the rate of glycolysis, but caused a decrease in the ATP content of the cells of the latter.

Bernheim (1972) proposed that phenol reacted primarily with the phospholipid component of the cell membrane of *Pseudomonas aeruginosa* subsequently causing an increase in the permeability of the cell membrane. Phenol was later shown to cause rapid swelling of *P.aeruginosa* cells (Bernheim 1974). *Pseudomonas fluorescens* and *P.fragi* were also found to leak intracellular UV-absorbing material and ^{14}C labeled compounds in the presence of BHA (butylated hydroxy-anisole). Significant changes were caused also in the fatty acid composition and phospholipid content of these

organisms (Davidson & Branen 1980).

Prindle and Wright (1977) reported that high concentrations of phenols precipitated all cellular proteins, whereas low concentrations selectively inhibited essential enzymes. It has been shown also that phenolic antioxidants bind proteins, most probably through hydrophobic interactions (Cornell 1979, Cornell *et al.* 1971). Degre & Sylvestre (1983) and Degre *et al.* (1983), studying the mode of action of BHA against *Staph.aureus* Wood 46, found that it affected the permeability of the membranes and acted as a reducing agent on the electron transport system of the microorganism, but did not stimulate endogenous respiration of *Staph.aureus* like other electron donors, rather it inhibited the process.

Rico Munoz *et al.* (1987) found that the phenolic compounds which they tested against *Staph.aureus* LP and A100 did not have any overall effect on the activity of the membrane-bound ATPase and concluded that phenolic compounds probably do not share a common mechanism of action and that there may not be a single target associated with inhibition of microorganisms by these compounds.

Recently, Ruiz Barba and Jimenez Diaz (1989) noted that brines from non-lye treated as well as those from heat-treated green olives inhibited not only the survival of *Lact. plantarum* strains but affected also the shape and size of the organism in such brines. Aqueous solutions of the total phenolics extracted from these brines as well as oleuropein extracted from green olives had the same bactericidal effect. Heat-treated oleuropein exhibited a strong bactericidal effect. Alkali-treated oleuropein did not. Indeed it allowed survival of most of the strains of *Lact. plantarum.* The bactericidal effect was accompanied by distortion of the typical bacillary structure as well as degradation of cell walls with concomitant loss of Gram stain reaction, and/or mesosomes in the membranes of *Lact. plantarum* (Ruiz-Barba *et al.* 1990, 1991).

Further biological properties

Many biological properties have been attributed to oleuropein, its derivatives as well as to phenolic substances in general. Pharmacological properties (as an hypotensive agent) have been attributed to oleuropein (Mazet 1938; Julius-Bijlsma 1961). Movsumov *et al.* (1987) suggested that the hypotensive effect of the extracts from olive leaves and fruits is mainly related to oleuropein. Kubo *et al.* (1985) refer to oleuropein as a probable phytoalexin precursor. Molluscicidal activity (LD^{50}=250ppm) of oleuropein against *Biomphalaria glabratus* was reported by Kubo and Matsumoto (1984b). Degradation products from the hydrolysis of oleuropein inhibit oviposition of *Dacus oleae*, an insect which infests Mediterranean olive trees. This has been attributed to the formation of 3,4-dihydroxyphenylethanol and to other phenolic substances present in the juice trickling from the oviposition wounds of *Dacus oleae*. These substances deter further oviposition on olives already infested (Girolami *et al.* 1975, 1981). Bongi (1986) noted that oleuropein inhibited flowering in *Kalanchoe blossfeldiana*, stimulated rooting in *Vigna radiata* cuttings, and irreversible stomatal closure in bioassays with *Commelina communis*. It inhibited growth but induced stomatal closure on small olive trees in pots and decreased olive callus growth to an appreciable extent.

In the following results section attention was directed at the effect of phenolic extract of olives and oleuropein on:

1) growth of *Staphylococcus aureus, Salmonella enteritidis, Enterococcus faecalis* and *Pseudomonas fragi*;

2) germination and outgrowth of the endospores of *Bacillus cereus*;

3) the fate of *Salmonella enteritidis* in mayonnaise, and

4) toxin and protein formation by *Staphylococcus aureus* in laboratory media or milk.

Four papers have been published on this work; offprints are presented in the Appendix.

MATERIALS AND METHODS

In Broths

1. Microbiological

Paper - disc bioassay

Paper discs (6.0mm, Whatman No 2017, 006) were soaked with different quantities of a filter sterilised (0.22μm Millipore) solution in water of oleuropein (Extrasynthese, 69730 Genay, France) or ethyl acetate extract from green olives (0.5g/10ml water, see Chapter 2, Fig. 2.9b) and placed on the surface of Plate Count Agar in Petri dishes inoculated with various microorganisms. After overnight incubation at 25°C, zones of inhibition were sought and photographed.

Bacterial strains

Staphylococcus aureus S-6, a producer of enterotoxin B, and *Staphylococcus aureus* 100, a producer of enterotoxin A, were used. Both strains were kindly provided by Dr H.S.Tranter and Prof. M.Bergdoll respectively. *Staphylococcus carnosus, Enterococcus faecalis, Lactobacillus plantarum, Bacillus subtilis, Salmonella enteritidis* and *Pseudomonas fragi,* taken from the culture collection of Bath University, were used also. *Lactobacillus plantarum* was maintained on slopes of MRS agar while all the rest mentioned cultures were maintained on slopes of Plate Count Agar at 4°C and subcultured every 4-6 weeks. *Bacillus cereus* T was kindly provided by Prof. G.W.Gould and maintained on slopes of Potato Dextrose Agar (PDA; pH: 7.2).

Growth media

Six different media were used:

a1. NZ Amine A medium (NZA) contained (g/l): N-Z amine A (Sheffield Chemical Co. Norwich, NY, USA), 40.0; yeast extract (Oxoid), 4.0; K_2HPO_4, 1.0;

a2. NZ Amine A medium (NZA) contained (g/l): NZ Amine A (Sheffield Chemical Co., Norwich, NY, USA), 20.0; proteose peptone No.3 (Difco Ltd.), 20.0;

b1. Brain Heart Infusion (BHI) medium contained (g/l):
BHI solids (Lab M, Bury, Lancs, UK) 17.5; tryptose (Lab M) 10.0; NaCl 5.0; Na_2HPO_4 25.0;

b2. Brain Heart Infusion broth (BHI; Oxoid Ltd.)

c. Tryptone Soya Broth (Lab M, code lab4).

d. Coliform Broth (Malthus Ltd. code 490-003)

The media were sterilised by autoclaving (121°C for 15min).

In some experiments glucose, sterilised by filtration (0.22 μm; Millipore), was used to supplement a medium at a final concentration of 0.2% (w/v). When the Malthus instrument was used, a filter-sterilised (0.22 μm, Millipore) growth-factor solution (1% w/v niacin and 0.5% w/v thiamin) was added to each medium to give a final concentration of 0.001% (w/v) and 0.0005% (w/v) respectively and 1% (w/v final concentration) glucose added immediately before inoculation.

Experimental procedure

A loopfull of culture of *Staphylococcus aureus* S-6, *St.aureus* 100, *Enterococcus faecalis* or *Pseudomonas fragi* from a slope of Plate Count agar

was used to inoculate 100ml of the appropriate medium selected for each experiment (NZA, BHI, TSB), without glucose in a screw-capped bottle (200ml). After overnight (18h) incubation at 37°C and 25°C for *Pseud.fragi*, 20-40 μl amounts of culture were used to inoculate 2x50 ml of a growth medium with or without glucose as well as with or without commercial oleuropein (0.5%w/v) or ethyl acetate extract of green olives (5ml of ethyl acetate extract -5%w/v- per 45ml of broth; final concentration 0.5%w/v) in bottles (50ml). These cultures were incubated statically for 24h at 37°C or 25°C for *Pseud.fragi*. At regular intervals samples (2ml) were removed aseptically from each culture and used immediately for growth determination by measuring the optical density at 600nm with a spectrophotometer. The remainder of each sample was frozen at -20°C until subsequent estimation of exoprotein.

Monitoring the growth

The turbidity of cell suspensions of *Staph. aureus* S-6 and 100, *Enterococcus faecalis* and *Pseudomonas fragi* in NZA (a1) medium was determined by measuring the optical density at 600nm using a SP6-550 UV/VIS Spectophotometer. Alternatively samples (0.15ml) of these cultures were used to fill six wells of a microtitre plate. Uninoculated wells were filled (0.2ml) with sterile distilled water in order to prevent desiccation of the inoculated ones. The plates were incubated statically for 24h at 37°C or 25°C for *Pseud.fragi*. The Dynatech MR600 microplate reader was used and the data analysed with a link BBC microcomputer at 550nm.

In some experiments with *Staph.aureus* S-6 and *Salmonella enteritidis*, the microbial growth was monitored with the Malthus 2000 instrument. In this case, the growth medium (NZA, BHI or Coliform Broth) was dispensed (2.5ml final volume) into sterile closed glass reaction cells containing platinum electrodes (Malthus Ltd.). Oleuropein (Extrasynthese, Genay,

France) was dissolved in distilled water (250 mg/ml). The suspension was warmed to 40-50°C to aid dissolution and the solution sterilised by filtration (0.22 μm; Millipore). This solution was added to reaction cells each containing growth medium (NZA, BHI or Coliform Broth) to give a final concentrations of 0-0.6% (w/v) oleuropein in an ultimate reaction volume of 2.5ml. The ethyl acetate extract of green olives was added in BHI to give also final concentration of 0-0.6% (w/v). These reaction cells were incubated at 37°C for 1h before inoculation. The cells were then inoculated with 0.1ml of an overnight culture of *Staph. aureus* S-6 or *Salm. enteritidis* grown at 37° C in the same growth medium (NZA, BHI or Coliform Broth). After inoculation the reaction cells were incubated statically in the water bath of a Malthus 2000 system maintained at 37°C. Changes in the conductance of the medium during growth were monitored every six minutes by the Malthus 2000. After 48h the reaction cells were sampled aseptically to determine the viable count (20μl of samples placed as drops on a dry surface of a Petri dish with Plate Count Agar and incubated for 24h at 37°C; Miles & Misra 1938) and enterotoxin concentration (see below).

In some experiments, the effect of oleuropein on actively growing cells was studied by the addition of sterile oleuropein solution (0.1, 0.2 and 0.4% w/v final concentration) or distilled water to Malthus reaction cells 6h after the inoculation of 2.5 ml medium with 0.2 ml of an overnight culture of *Staph. aureus* S-6.

Statistical analysis

In order to examine the effect of pH in combination with the 0.1% and 0.6% (w/v) of oleuropein on three different inoculum sizes of *Staph. aureus*

S-6 or *Salmonella enteritidis*, a three way analysis of variance experiment was designed. NZA or Coliform Broth were adjusted to pH values (8, 7, 6, 5) using phosphate buffers (NaH_2PO_4 - Na_2HPO_4) before sterilisation. The pH of the media did not change after sterilisation. The final growth (expressed as microsiemens) after 48h at 37°C and the detection time (expressed as hours) of these organisms were collected from the reaction cells of the Malthus Instrument and analysed by analysis of variance with the SYSTAT (Systat Inc., Evanston, IL, USA). When NaCl was used in combination with oleuropein against *Salm.enteritidis*, it was added in a concentration of 0.5% (w/v) with or without 0.2 or 0.5% (w/v) oleuropein.

Production and cleaning of spores of *Bacillus cereus*

Bacillus cereus T was grown at 30°C on PDA (Potato Dextrose Agar; pH: 7.2). When sporulation was complete and the sporangia had lysed (24h), the spores were washed off the agar with ice-cold distilled water, washed a further six times and harvested by centrifugation (4000 x **g**). Suspensions were cleaned of vegetative cells and debris by discarding the uppermost layers of the pellets obtained by centrifugation. The clean spore suspensions were stored at -20°C (Gould 1964; Tranter & Board 1982).

Germination of *Bacillus cereus*

A spore suspension (0.02 ml; 10^9 - 10^{10} cfu) was diluted in 3ml of phosphate buffer (Na_2HPO_4-KH_2PO_4; pH 6.8) and activated by heating in a water bath at 70°C for 30 min. The spores were then separated from the phosphate buffer by centrifugation (4000 x **g**), and added to 2ml of TSB (Tryptone Soya Broth; Lab M, code lab4). L-Alanine and inosine were added in final concentrations of 10 and 1mM, respectively, and the optical density (OD) at 580 nm was measured with a Philips Pye Unicam PU 8650

spectrophotometer in a room at 30°C. Immediately and at regular intervals after inoculation, a 0.1 ml sample was removed from the cuvette and mixed with 0.4 ml of formaldehyde-saline (2% w/v) to arrest spore germination and outgrowth. A drop of the suspension was placed on a clean microscope slide, viewed with a phase-contrast microscope, and photographed. Three hundred spores, present in 10 randomly selected fields of view, were scored as phase bright or dark and the ratio of germinated (dark) to ungerminated (bright) spores expressed as a percentage. Various amounts of oleuropein or phenolic extracts from olives were added at different times to a spore suspension of *B.cereus* in TSB. Because of changes due to oxidation of coloured extracts, TSB with addition of the same amounts of the extracts was used as the control to monitor colour changes. Once changes in the optical density had stopped (*ca* 90 min from the beginning of germination), decimal dilutions made from the samples in the cuvettes were inoculated on PDA (pH 7.2) in Petri dishes. After a 24-h incubation at 30°C, the numbers of colonies were counted.

2. Physicochemical

Exoprotein estimation

The method of Sedmak and Grossberg (1977) was used to determine the secretion of exoprotein using bovine serum albumin (Sigma) as standard.

Glucose and L-lactic acid determination

The concentrations of L-lactic acid and glucose in samples of supernatant fluids were estimated by the lactate dehydrogenase reaction (Gutman & Wahlefeld 1974) and the GOD-Perid kit (Boehringer, Mannheim, GmBH) respectively.

SDS Polyacrylamide Gel Electrophoresis

The protein profiles of uninoculated and inoculated growth medium were determined by sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis on gradient gels as described in the LKB laboratory manual for the Midget Electrophoresis Unit. Samples were freeze-dried (Morgan freeze-dryer, Modulo). After staining (with 0.1% w/v Coomasie blue R-250 in 25% v/v methanol and 10% v/v acetic acid), gels were destained for 3h in 40% (v/v) methanol and 10% (v/v) acetic acid. The destained gels were scanned (chromoscan 3, Joyce Loebl, Vickers plc in England or Hellena, France for samples examined in Greece) and traces obtained (Nychas *et al.* 1990).

HPLC analysis of proteins

The protein profiles of uninoculated and inoculated growth media were analysed in a Spectra Physics High Performance Liquid Chromatography consisting of a Spectra Physics P2000 two pump system, connected with a Spectra Focus forward optical scanning detector and a MZ-SIL (250 x 4mm) 300 C18 7um column. Buffer A was 10mM potassium phosphate, pH 7.2, in distilled water and Buffer B was acetonitrile and buffer A (60:40 v/v). Linear gradient was used from 0% to 7% buffer B in 20min with a flow rate of 0.5 ml/min. Peak width was 12, peak threshold 600 and 0.068 AUFS at 280nm.

Enterotoxin assay

The production of enterotoxin B in the presence or absence of oleuropein was estimated by the reversed passive latex agglutination (RPLA) kit

(Unipath Ltd.) according to the manufacturer's instructions. For this a sample (1ml) was removed from the reaction cells and centrifuged (10,000 x g) for 5 min to remove the cells. The supernatant fluid was removed and stored at -80°C prior to use. Although the kit is not able to give an accurate quantitative measurement of the toxin present in culture fluids, the amount of toxin present was estimated according to the equation: SEB/ml of original solution = detection limit x 2^{n-1}, where the detection limit according to the manufacturer is 0.5ng and n is the number of the well in which the endpoint of a given sample is found (Salomon & Tew 1968).

Electron microscopy

The JEOL JSM-T330 Scanning Microscope was used. The spores and bacteria were fixed with 5ml of fixative, 1ml of glutaraldehyde, 2.5ml cacodylate buffer 0.1M and 1.5ml distilled water. They were resuspended in 1ml fixative in an Eppendorf tube and left to fix in the fume cupboard for 2 h. Then the cells were washed free of the fixative with 0.1M cacodylate buffer (3 times) and resuspended in OsO_4 (1% in cacodylate buffer). They were left for 1 h and then the OsO_4 eliminated by centrifugation. The cells were left overnight in cacodylate buffer (0.1 M). Next day they were dehydrated using an acetone series, 30% (v/v) 2 x 10 mins; 50% (v/v) 2 x 10 mins; 70% (v/v) 2 x 10 mins; 100% (v/v) 4 x 10 mins or change over 1h. The cells were air dried and gold coated in vacuum in an Edwards Sputter Coater S 150 B (Tranter 1982).

In Model Food System

Bacterial strains

Staphylococcus aureus S-6 and *Salmonella enteritidis* PT4 -kindly provided by Dr.T.Humphrey (Exeter PHLS)- were maintained on slopes of Plate Count Agar at 4°C and subcultured every 4-6 weeks. An overnight culture (18h) was grown in 50ml nutrient broth (Lab M) at 37°C, harvested by centrifugation and washed twice in saline to give a final cell density of 10^9 cells/ml.

Milk system

The model milk system consisted of a nonfat milk (kindly provided by Prof.C.Kechagias). Solids suspension (10% w/v) was prepared by rehydrating the nonfat milk solids in deionised water and autoclaving at 115°C for 15min. The milk was dispensed (2.5ml final volume) into sterile Malthus glass reaction cells as in the case of culture media described above and inoculated with 0.1ml of an overnight culture of *Staph. aureus* S-6 grown at 37°C in NZA. Sterile oleuropein was added at final concentrations of 0.5, 1, 1.5 and 2% (w/v) and the cells incubated in the Malthus water bath at 37°C.

Mayonnaise preparation

Mayonnaise (300ml oil; 2 egg yolks (size 2); 9ml (6%) acetic acid; final pH 4.3) was prepared by whisking two egg yolks with an electric hand mixer (Carlton AMO3) and gradually adding the oil (150ml) with a sterile Pasteur pipette during continuous mixing. Six ml of acetic acid were whisked in to thin down the mixture so that it did not curdle. The remaining oil (150ml) was then added and finally another 3ml of acetic acid. The final pH, measured with a pH meter (EIL 7050), was 4.3. The following oils were used: sunflower, a proprietory blend of olive oils from EEC countries and extra virgin olive oils from Italy and Greece. All were purchased from

supermarkets except of the Greek extra virgin olive oil which was provided by Elais S.A.

Inoculation

About 5 x 10^4cfu/g *S. enteritidis* PT4 were thoroughly mixed with 40g of mayonnaise by extensive stirring with a spatula. Uninoculated samples were used as controls. Samples were incubated at 20°C. The experiments were done four times with seven replicates per oil on one occasion and four in a further three trials.

Bacterial enumeration

At 0, 24, 48 and 72h samples were diluted in 1/4 Ringer's solution, 0.1 ml of an appropriate dilution was spread on nutrient agar in Petri dishes and colonies counted after 24h at 37°C.

RESULTS

In Broth

Effect of oleuropein and olive extract on various spoilage and food borne bacteria

General

The effect of commercial "pure" oleuropein as well as an ethyl acetate extract from green olives (see Chapter 2, Fig. 2.9b & 2.10a) and olive oil (Fig. 2.10b) on *Pseudomonas fragi* (A, Fig. 3.1), *Lactobacillus plantarum* (B, Fig. 3.1), *Staphylococcus carnosus* (A, Fig. 3.2), *Staphylococcus aureus* (B, Figs. 3.2 & 3.3), *Enterococcus faecalis* (B, Fig. 3.4), *Bacillus subtilis* (Fig. 3.5) and *Bacillus cereus* T (Figs 3.6 & 3.7) was examined using the paper disc technique.

In all cases a clear zone of inhibition was evident in the bacterial lawn after 24h incubation at 25°C. Figure 3.1 shows inhibition of *Pseudomonas fragi* (A) and *Lactobacillus plantarum* (B) when the paper discs contained an ethyl acetate extract of green olives. With *Staphylococcus carnosus* (A, Fig. 3.2) the zone of inhibition was not so large as it was with *Staphylococcus aureus* (B, Fig. 3.2). A clear zone was also evident with *Enterococcus faecalis* (Fig. 3.4) with all dilutions of olive extract. Growth inhibition of *Staph.aureus* was seen when the paper discs were soaked in commercial oleuropein (Fig. 3.3). With *Bacillus* spp. the zone of inhibition was related to the concentration of the ethyl acetate extract of green olives used, as is evident with *B.subtilis* (Fig. 3.5) and *B.cereus* T (Figs 3.6 & 3.7). There was also inhibition of growth of *B.cereus* T when phenolic compounds extracted with methanol/water from olive oil were used. The zones around

Fig. 3.1. Zones of inhibition of *Pseudomonas fragi* (a) and *Lactobacillus plantarum* (b) by ethyl acetate extract of green olives

a b

Fig. 3.2. Zones of inhibition of *Staphylococcus carnosus* (a) and *Staphylococcus aureus* (b) by ethyl acetate extract of green olives

a b

Fig. 3.3. Zones of inhibition of *Staphylococcus aureus* by commercial oleuropein 0.2% (a) and 0.4% w/v (b)

a b

Fig. 3.4. Zones of inhibition of *Enterococcus faecalis* by ethyl acetate extract of green olives

Fig. 3.5. Zones of inhibition of *Bacillus subtilis* by different amounts of commercial oleuropein (0.2 and 0.4% w/v)

Fig. 3.6. Zones of inhibition of *Bacillus cereus* T by different amounts of ethyl acetate extract of green olives

Fig. 3.7. Zones of inhibition of *Bacillus cereus* T by different amounts of phenolic extract of virgin olive oil (Fig. 2.10b)

the paper discs containing oil extract (Fig. 3.7) however were narrower than those caused by the olive extract (Fig. 3.6).

The growth of *Pseudomonas fragi*

The effect of commercial "pure" oleuropein (0.5% w/v) and the ethyl acetate green olive extract (0.5% w/v; Fig. 2.9b) on *Pseudomonas fragi* in NZA (a1) medium is shown in Figure 3.8. In both cases the absorbance (optical density) at 550nm of a medium without oleuropein or olive extract was always significantly higher than in that containing one or other of the above.

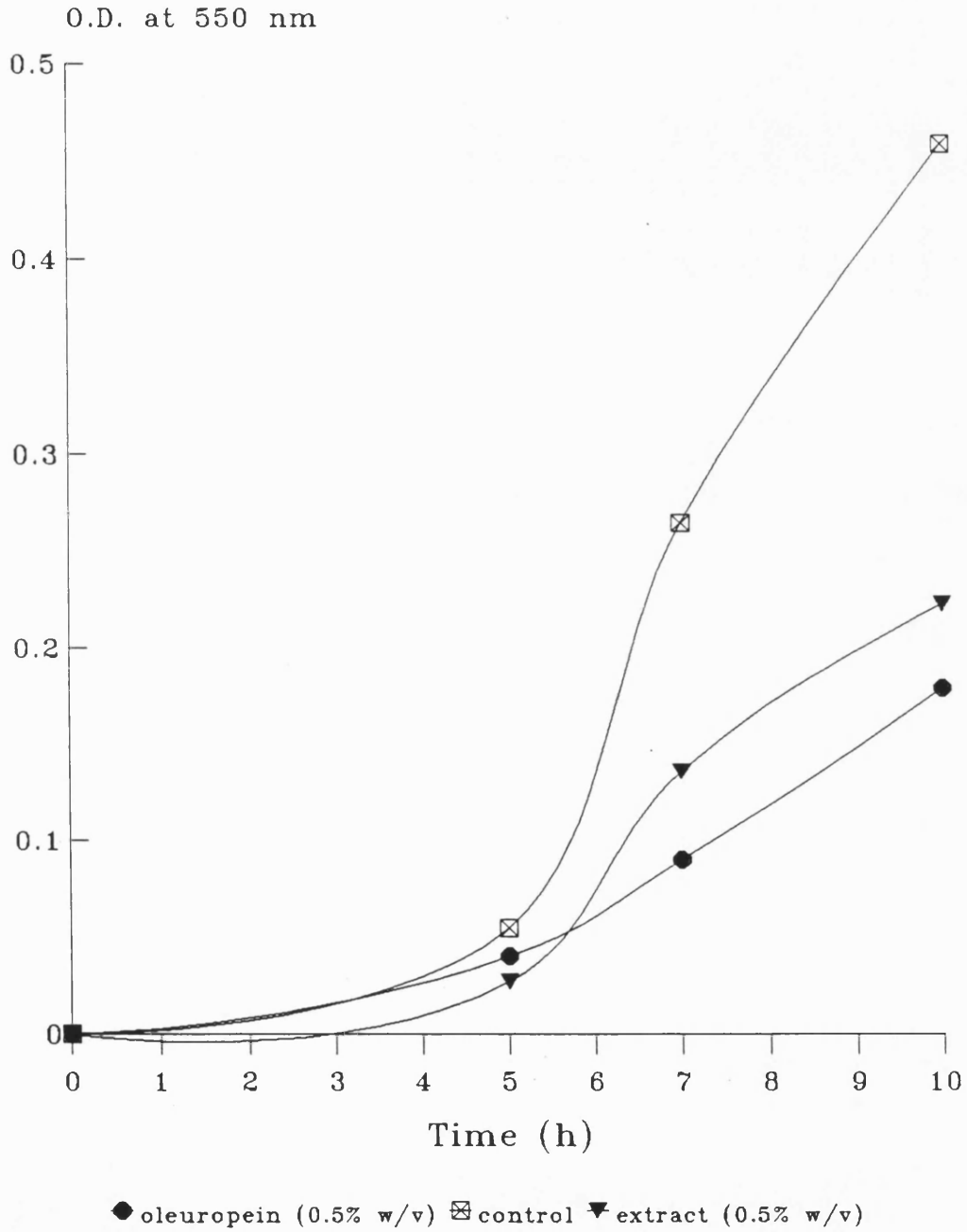
The growth of *Enterococcus faecalis*

Results similar to those discussed above were obtained when *Ent.faecalis* was studied. The addition of commercial oleuropein (0.5% w/v) or ethyl acetate green olive extract (0.5% w/v; Fig. 2.9b) in NZA (Fig. 3.9) retarded the growth of this organism compared to that in the unsupplemented medium.

The growth of *Staphylococcus aureus* S-6

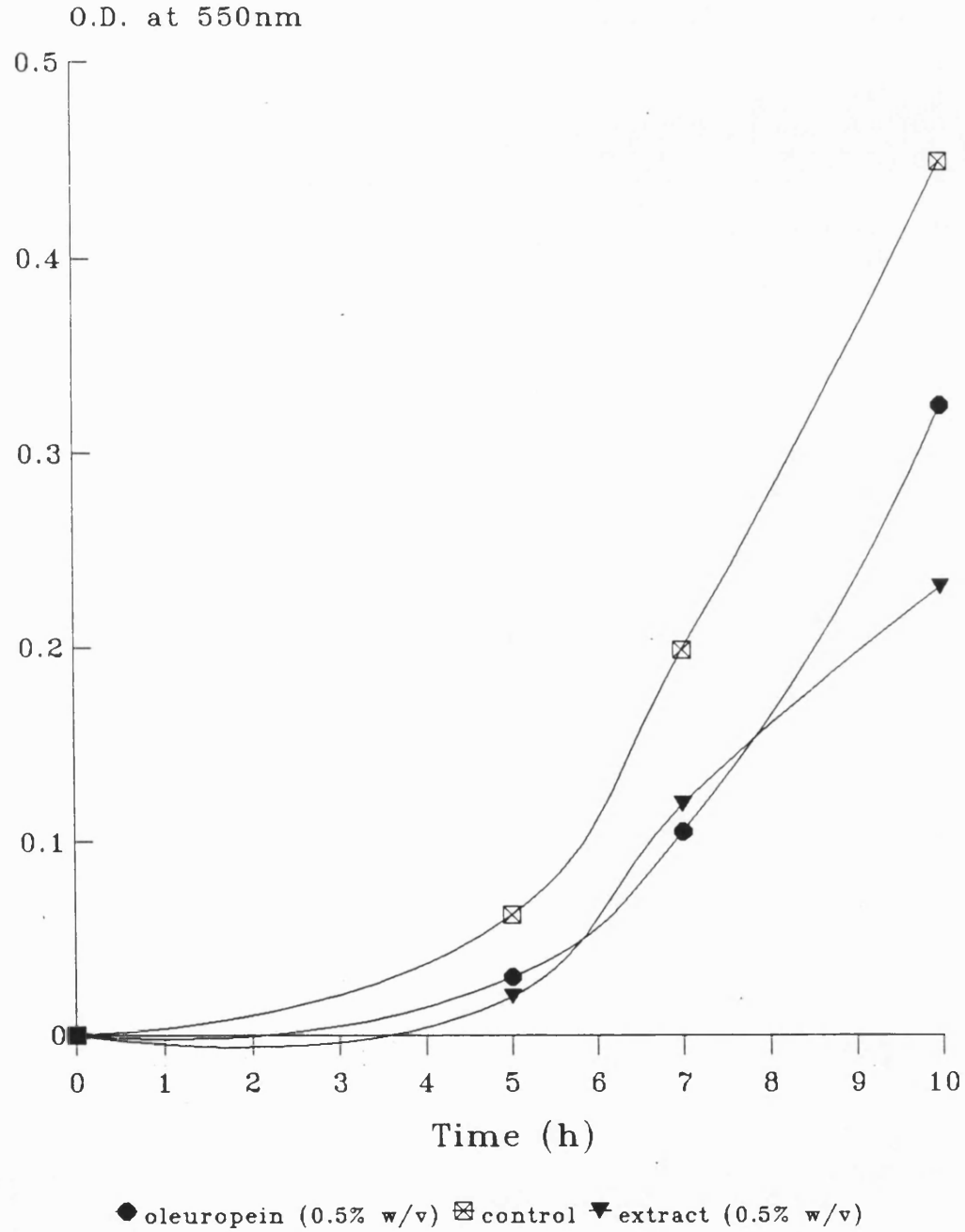
Figure 3.10 shows the optical density values (at 550nm) when *Staph. aureus* was grown in NZA with or without the addition of phenolic extract from olive oil. The two olive oil extracts (oil 1; Greek fresh extra virgin & oil 2; Italian virgin olive oil) differed in the content of phenolic compounds as was evident from their analysis with HPLC (Fig. 2.11a,b). The first olive oil, which contains much more phenolics than the second one, had a dramatic effect on *Staph.aureus* and inhibited completely any increase in the optical density thus inhibiting microbial growth even in concentration of 0.5% (v/v). The second extract from olive oil No.2 was less active than the

Fig. 3.8. The effect of ethyl acetate extract of green olives and commercial oleuropein on *Pseudomonas fragi* at 25°C in NZA



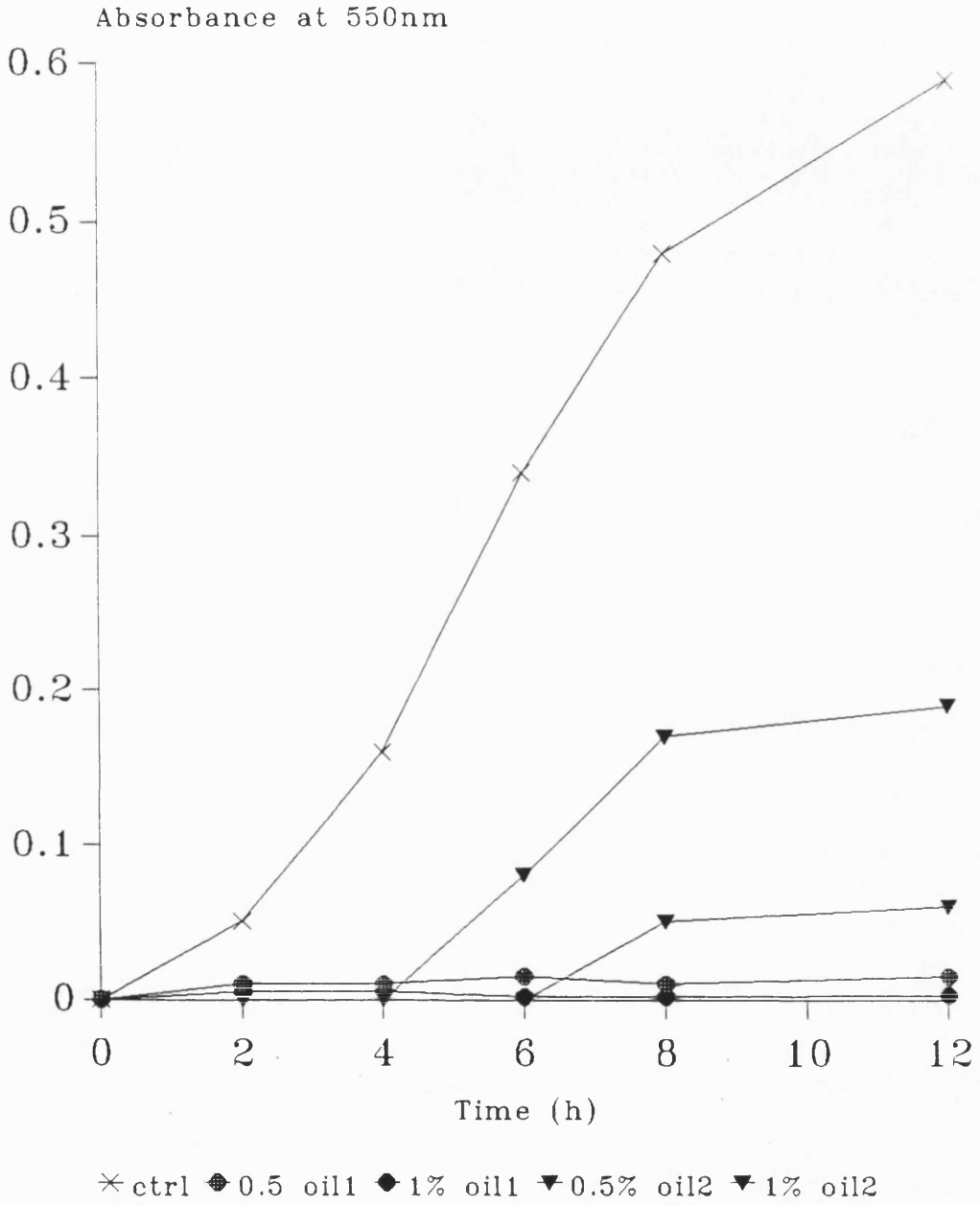
Each point average of four observations

Fig. 3.9. The effect of ethyl acetate extract of green olives and commercial oleuropein on *Enterococcus faecalis* in NZA at 37°C



Each point average of four observations

Fig. 3.10. The effect of phenolic compounds extracted from two virgin olive oils on the growth of Staphylococcus aureus S-6 at 30°C

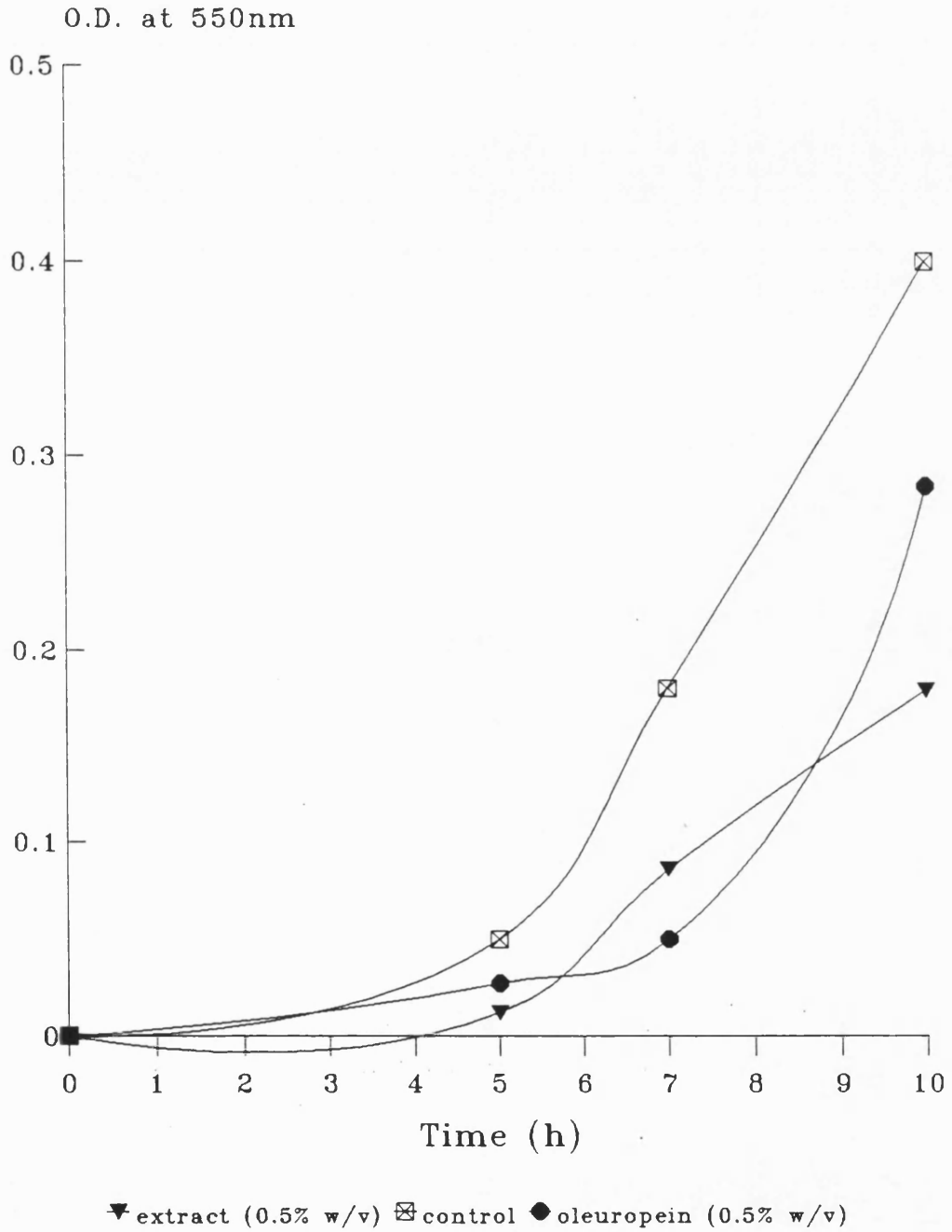


Each point the average of 3 experiments
oil 1: Greek extra virgin olive oil (Fig. 2.11a)
oil 2: Italian extra virgin olive oil (Fig. 2.11b)

previous one but it also delayed and decreased markedly the optical density especially in higher concentrations.

Preliminary experiments with microtitre plates showed that *Staph.aureus* was inhibited by commercial "pure" oleuropein (0.5% w/v) or ethyl acetate extract of green olives (0.5% w/v; Fig. 3.11). The optical densities obtained after 11h of incubation at 37°C (Table 3.4) and the changes in the conductance (Figs 3.12 & 3.13) - when the growth of *Staph. aureus* S-6 was studied by the Malthus - were greater in NZA than in BHI medium even though the detection times (2.2h and 2.7h respectively, Table 3.5) and shape of the growth curves were essentially similar. This increased conductivity of NZA did not appear to be due to higher numbers of organisms as viable counts of this medium and BHI were almost identical after 48h incubation (Table 3.5). Initially there was a rapid change in conductivity corresponding to the logarithmic growth phase followed by a second much slower increase in conductivity which was slightly more pronounced in BHI than NZA. The presence of oleuropein in either medium at the time of inoculation resulted in an increase in both the lag and growth phases of *Staph. aureus*. These effects appeared to be concentration dependent (Figs. 3.12, 3.13). Growth was completely inhibited by concentrations of oleuropein of $>=0.4\%$ (w/v) in either medium; lower concentrations (0.1% w/v) delayed both the onset and extent of growth of the organism (Figs. 3.12, 3.13, Table 3.5). An intermediate concentration (0.2% w/v) completely inhibited the growth of *Staph. aureus* in BHI but allowed growth to occur in NZA despite a long delay in the lag period (approximately 30h). The Malthus system gave detection times for organisms in the presence of higher concentrations of oleuropein (Table 3.5) and, although there was a small increase in the conductivity (approximately 50mS) of these samples over 48h (Figs 3.12, 3.13), there was clearly no demonstrable growth. As long detection times were occasionaly recorded in uninoculated medium such observations may

Fig. 3.11. The effect of ethyl acetate extract of green olives and commercial oleuropein *on Staphylococcus aureus* S-6 in NZA at 37°C



Each point average of four observations

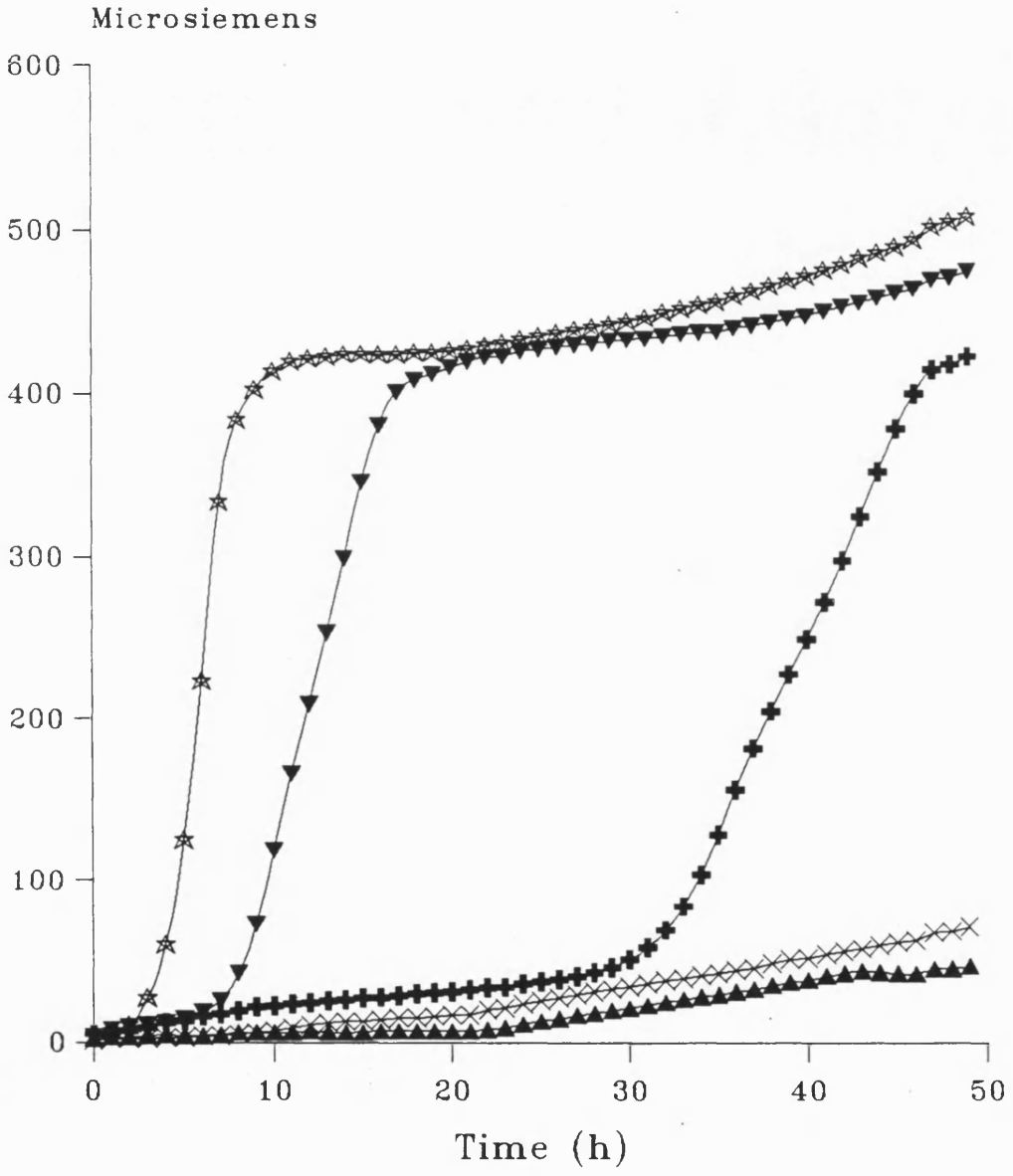
Table 3.4. The effect of phenolic compounds extracted from green olives and different media on growth and exoprotein formation by *Staphylococcus aureus* S-6 with incubation at 37°C

	Treatment	Bacterial density (O.D.600nm)	Exoprotein (μg/ml)	
Type of medium	Addition of olive extract (0.5% w/v)	Incubation time 11h	Incubation time 0h	Incubation time 11h
NZA*	+	0.208	12.5	152
	-	0.502	12.5	100
BHI$	+	0.040	92.0	150
	-	0.125	92.0	105

* : NZ Amine A medium (a1)

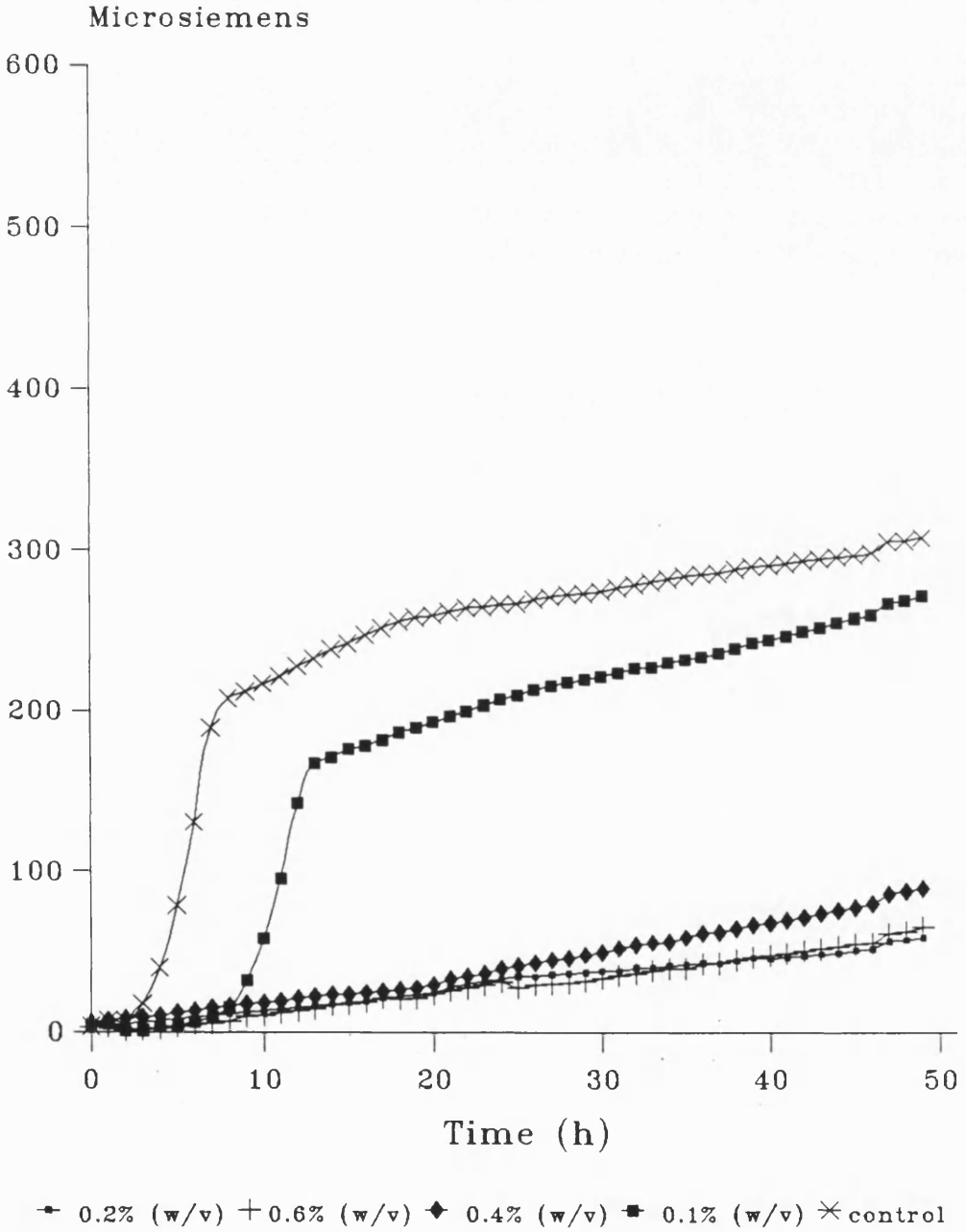
$: Brain Heart Infusion medium (b1)

Fig. 3.12. Growth of *Staphylococcus aureus* S-6 with or without the addition of different amounts of commercial oleuropein in NZA at 37°C in a Malthus Instrument



Each point average of four observations

Fig. 3.13. Growth of *Staphylococcus aureus* S-6 with or without the addition of different amounts of commercial oleuropein in BHI at 37°C in a Malthus Instrument



Each point average of four observations

Table 3.5. Growth of *Staphylococcus aureus* S-6 in NZA (a2) and BHI (b2) as monitored with a Malthus instrument and the effect of commercial oleuropein on detection time, glucose utilisation and production of L-lactate and enterotoxin B after 48h at 37°C

Growth medium	Oleuropein concentration (% w/v)	Detection time (h)	Viable count per 2.5ml	Total toxin (ng)	Glucose[&] concentration (mg/100ml)	Lactate[$] production (mg/100ml)
NZA[*]	-	2.2	9.1×10^8	80.0	26.5	0.82
NZA	0.1	7.0	6.5×10^8	10.0	57.8	0.72
NZA	0.2	30.8	5.3×10^8	0.25	278.0	0.27
NZA	0.4	46.5	<500	0.13	468.0	0.19
NZA	0.6	46.5	<500	0.13	680.0	0.20
BHI[*]	-	2.7	7.8×10^8	80.0	15.0	1.39
BHI	0.1	1.5	1.2×10^9	80.0	64.0	1.53
BHI	0.2	46.5	1.3×10^5	2.0	261.0	0.25
BHI	0.4	46.5	<500	1.0	310.0	0.25
BHI	0.6	23.0	<500	1.0	925.0	0.25

*: Initial inoculum level in NZA = 6×10^5 cells/2.5ml and BHI = 3×10^6 cells/2.5ml

&: Initial glucose concentrations were estimated to be 1036.5mg/100ml and 1153.2mg/100ml in NZA and BHI, respectively

$: Initial lactate concentrations were estimated to be 0.21mg/100ml and 0.15mg/100ml in NZA and BHI respectively

have been the result of small fluctuations in the medium baseline.

The addition of glucose to NZA increased its turbidity with incubation (Table 3.6). The addition of ethyl acetate extract of green olives or the commercial (pure) oleuropein, to NZA or BHI retarded staphylococcal growth (Tables 3.4, 3.5 & Figs 3.12, 3.13, 3.14). The same effect was observed in NZA even when the olive extract was used in combination with glucose addition (Table 3.6). Olive extract affected markedly the rate of growth of another strain of *Staph. aureus,* 100 in BHI (results not shown).

Although the presence of oleuropein in NZA or BHI at the time of inoculation delayed the onset of and the extent of the eventual growth of *Staph. aureus*, its addition to NZA midway through the initial growth phase did not appear to affect growth as demonstrated by change in conductivity (Fig 3.15) or the final cell counts in either medium (Table 3.7). However conductance values in the presence of oleuropein were slightly lower (407, 461 mS) compared to inoculated medium to which no oleuropein had been added (458mS).

The size of the initial inoculum, the amount of oleuropein and the pH of the medium influenced the growth of *Staph.aureus* (Table 3.8). Indeed the three way analysis of variance showed that the final growth (as indicated by microsiemens) or the detection time was affected by all the above mentioned factors (Table 3.8). The final population size of *Staph.aureus* was larger at pH 8 than at pH 5 in samples inoculated with the same inoculum size and at the same concentration of oleuropein (Fig. 3.16, 3.17 & 3.18). Similar results were found when the detection times were analysed (Figs 3.19, 3.20, 3.21).

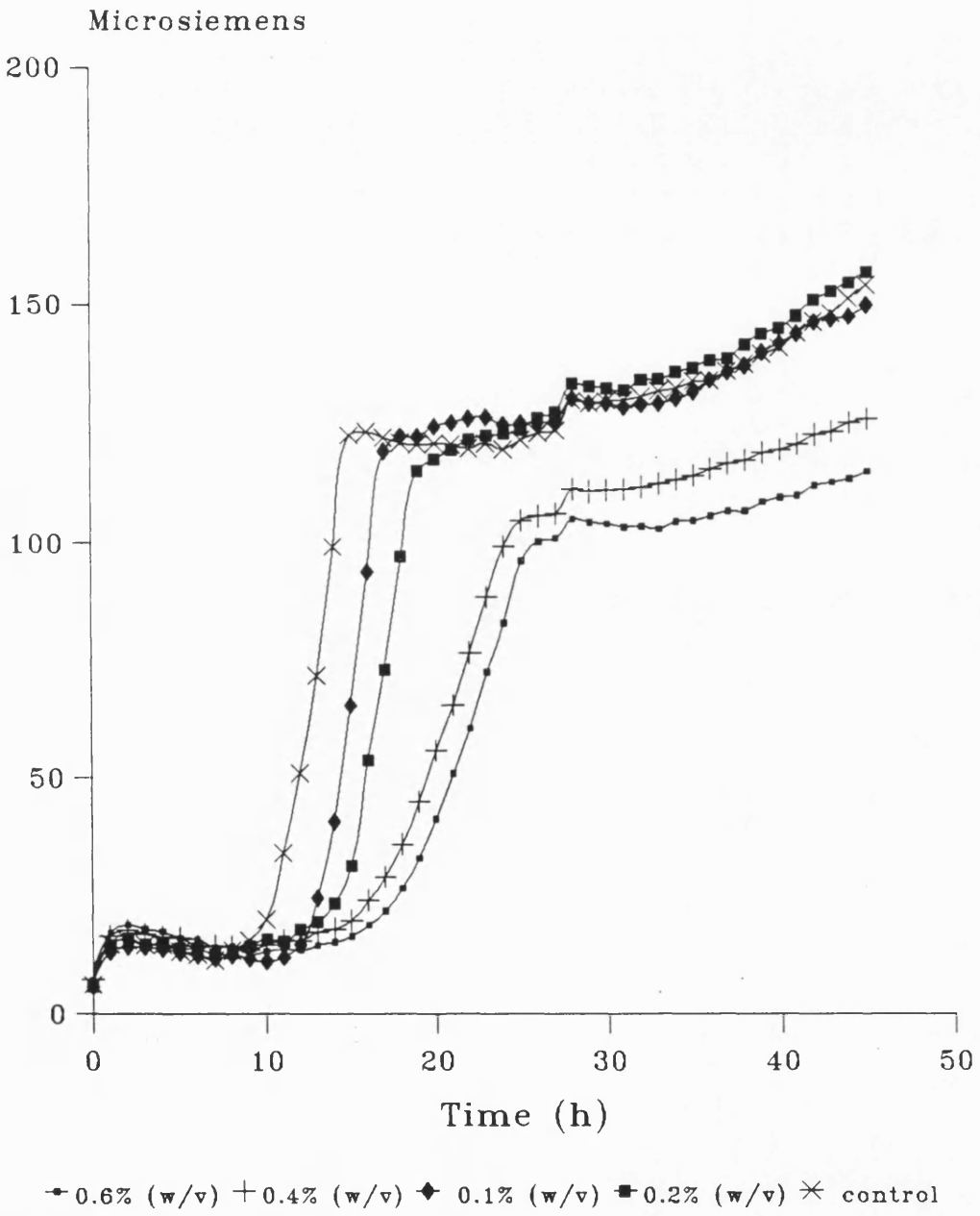
Effect of oleuropein on toxin production

The effect of oleuropein on the viable cell counts and toxin production in

Table 3.6. The effect of glucose and phenolic compounds extracted from green olives on growth and exoprotein formation by *Staphylococcus aureus* S-6 in NZ Amine A medium (a1)

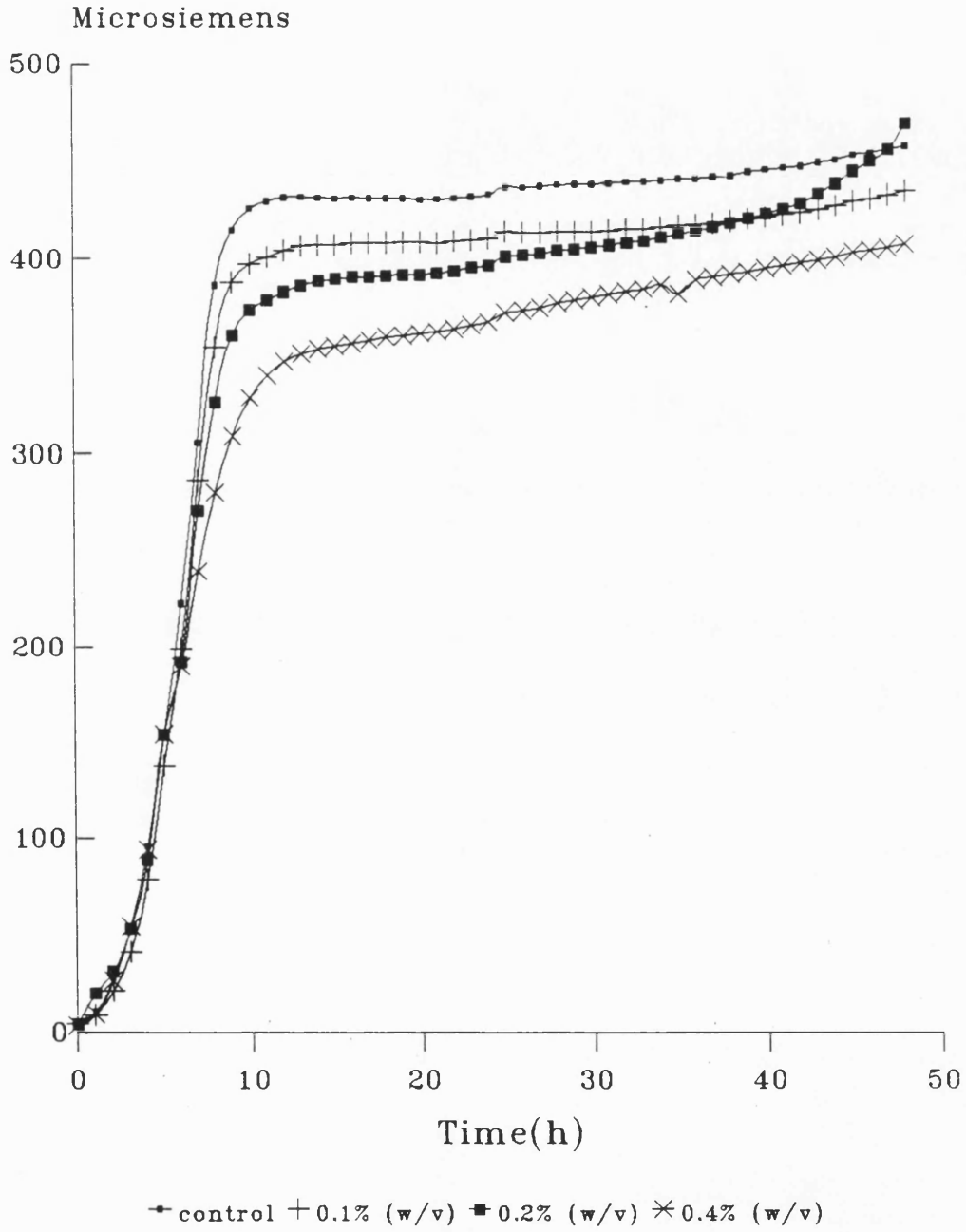
Addition to NZ Amine A medium		Bacterial density (O.D.600nm)	Exoprotein (μg/ml)	
Extract 0.5% (w/v)	Glucose 0.2% (w/v)	Incubation time 9h	Incubation time 0h	9h
+	-	0.120	12	52
+	+	0.420	12	32
-	-	0.490	12	47
_	+	0.680	12	39

Fig. 3.14. The effect of ethyl acetate extract of green olives on the growth of *Staphylococcus aureus* S-6 in BHI at 37°C in a Malthus Instrument



Each point average of four observations

Fig. 3.15. The effect of commercial oleuropein added in logarithmic phases of growth of *Staphylococcus aureus* S-6 in NZA at 37°C in a Malthus Instrument



Each point average of four observations

Table 3.7. The effect of the addition of commercial oleuropein (0, 0.1, 0.4, 0.6% w/v) midway through the initial growth phase of *Staphylococcus aureus* S-6 in NZA medium (a2) in a Malthus Instrument, on its detection time, and on toxin production, after 48 h of incubation (static) at 37°C.

Oleuropein (% w/v)	Detection time (h)	Viable cell count per 2.5ml	Total toxin produced (μg/ml)
0	4.9	9.2×10^8	1
0.1	5.5	9.0×10^8	1
0.4	6.5	9.1×10^8	1
0.6	6.2	8.9×10^8	0.5

Table 3.8. The effect of initial inoculum size, amount of commercial oleuropein and the pH of the medium on the final growth (a) and on the detection time (b) of *Staphylococcus aureus* S-6 in NZA (a2) at 37°C (Three way analysis of variance)

a

Source	df	f-ratio	Probability
(A) Inoculum size	2	60025	0.0000
(B) Oleuropein	2	100435	0.0000
(C) pH	3	331088	0.0000
A x B	4	11881	0.0000
A x C	6	16386	0.0000
B x C	6	22572	0.0000
A x B x C	12	12652	0.0000

b

Source	df	f-ratio	Probability
(A) Inoculum size	2	43643	0.0000
(B) Oleuropein	2	28978	0.0000
(C) pH	3	35314	0.0000
A x B	4	4501	0.0000
A x C	6	9514	0.0000
B x C	6	17349	0.0000
A x B x C	12	15035	0.0000

Inoculum size : $\log_{10}$7.15/ml, $\log_{10}$6.15/ml, $\log_{10}$5.15/ml
Oleuropein concentration: 0, 0.1, 0.6% (w/v)
pH : 5, 6, 7, 8

Fig. 3.16. The effect of pH and inoculum size on the final growth of *Staphylococcus aureus* S-6 in a Malthus Instrument (Inoculum size 1.00: $\log_{10}$7.15/ml; 0.10: $\log_{10}$6.15/ml; 0.01: $\log_{10}$5.15/ml)

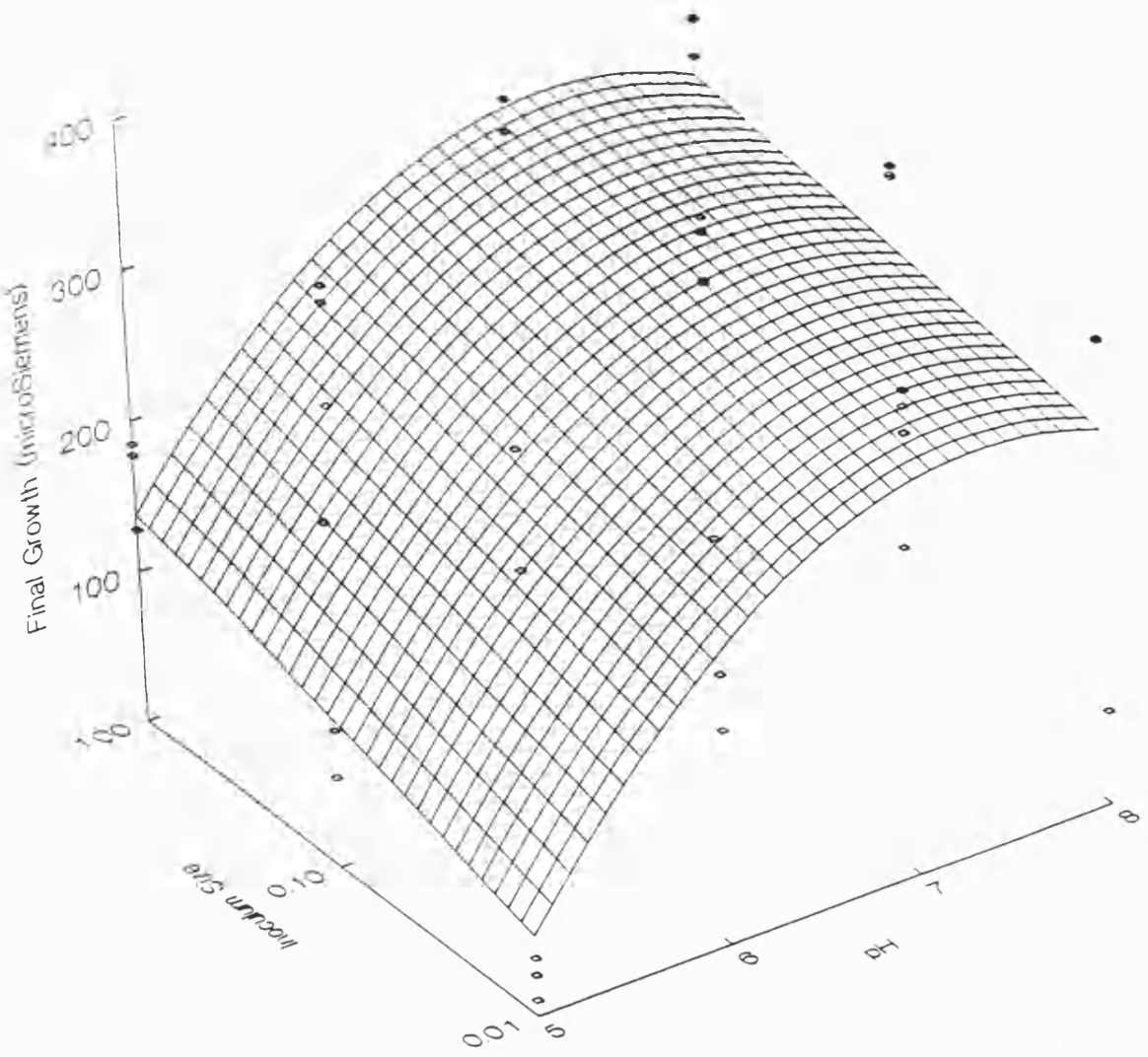


Fig. 3.17. The effect of commercial oleuropein concentration (% w/v) and inoculum size on the final growth of *Staphylococcus aureus* S-6 in a Malthus Instrument (Inoculum size 1.00: $\log_{10}$7.15/ml; 0.10: $\log_{10}$6.15/ml; 0.01: $\log_{10}$5.15/ml)

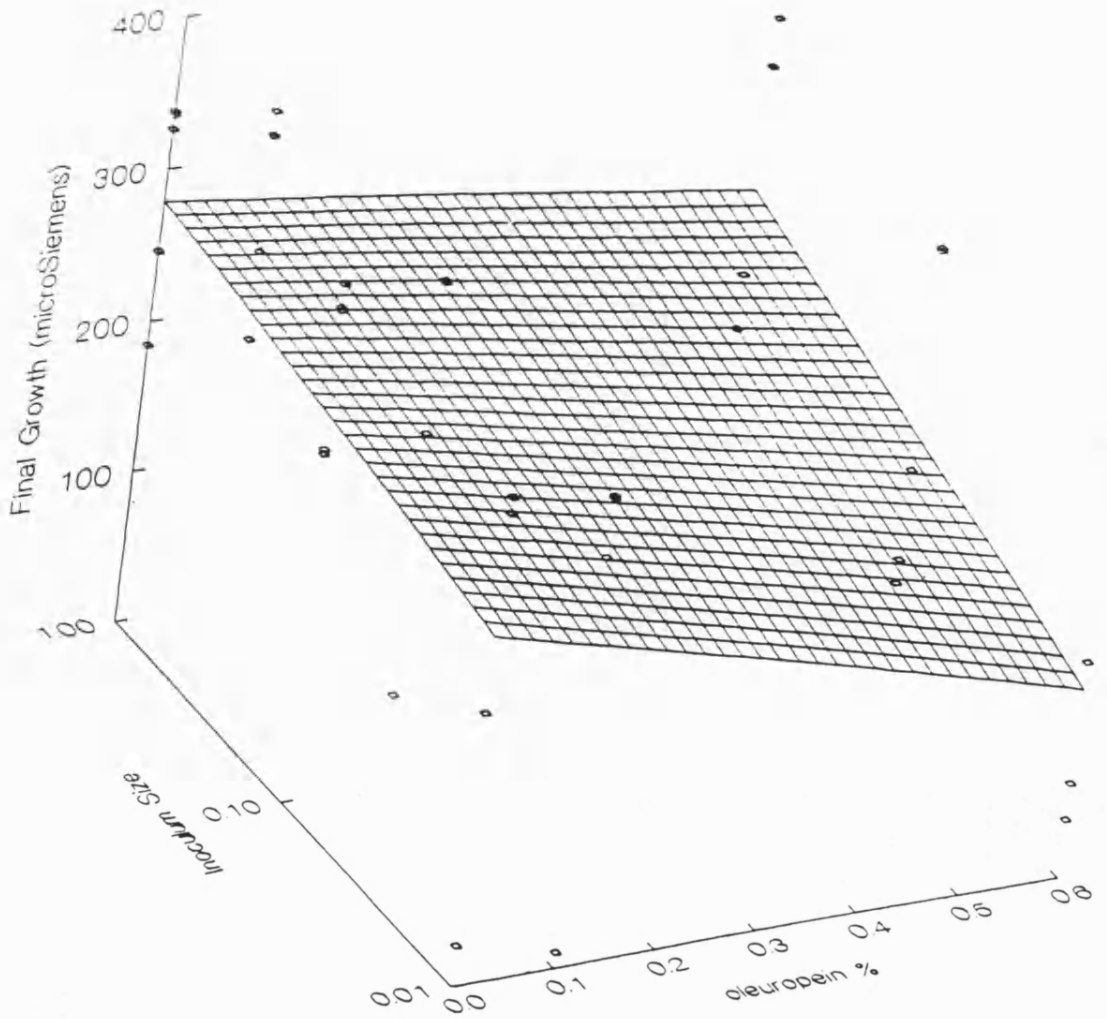


Fig. 3.18. The effect of pH and commercial oleuropein concentration (% w/v) on the final growth (conductance values) of *Staphylococcus aureus* S-6 in a Malthus Instrument

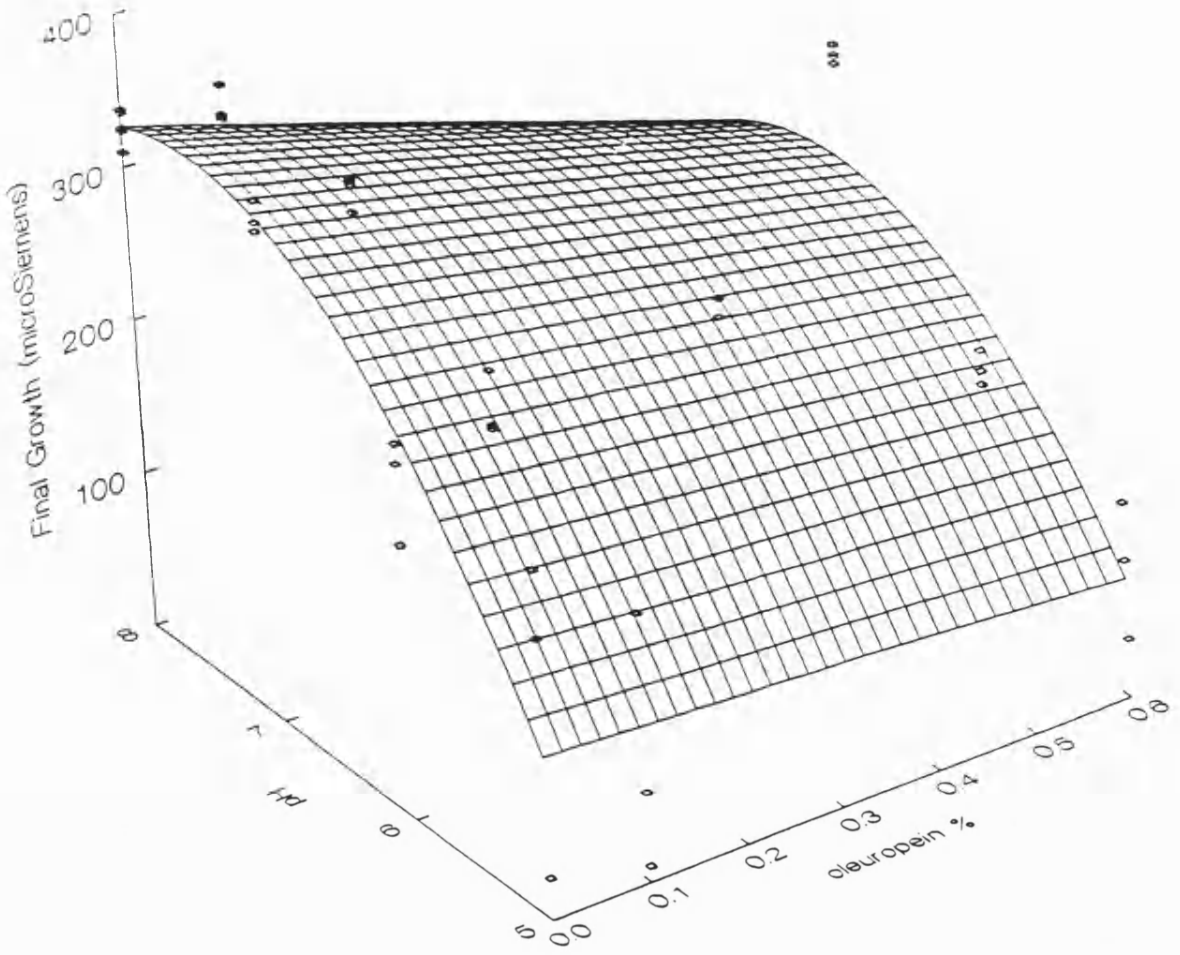


Fig. 3.19. The effect of commercial oleuropein concentration (% w/v) and inoculum size on the detection time of *Staphylococcus aureus* S-6 in a Malthus Instrument (Inoculum size 1.00: $\log_{10}$7.15/ml; 0.10: $\log_{10}$6.15/ml; 0.01: $\log_{10}$5.15/ml)

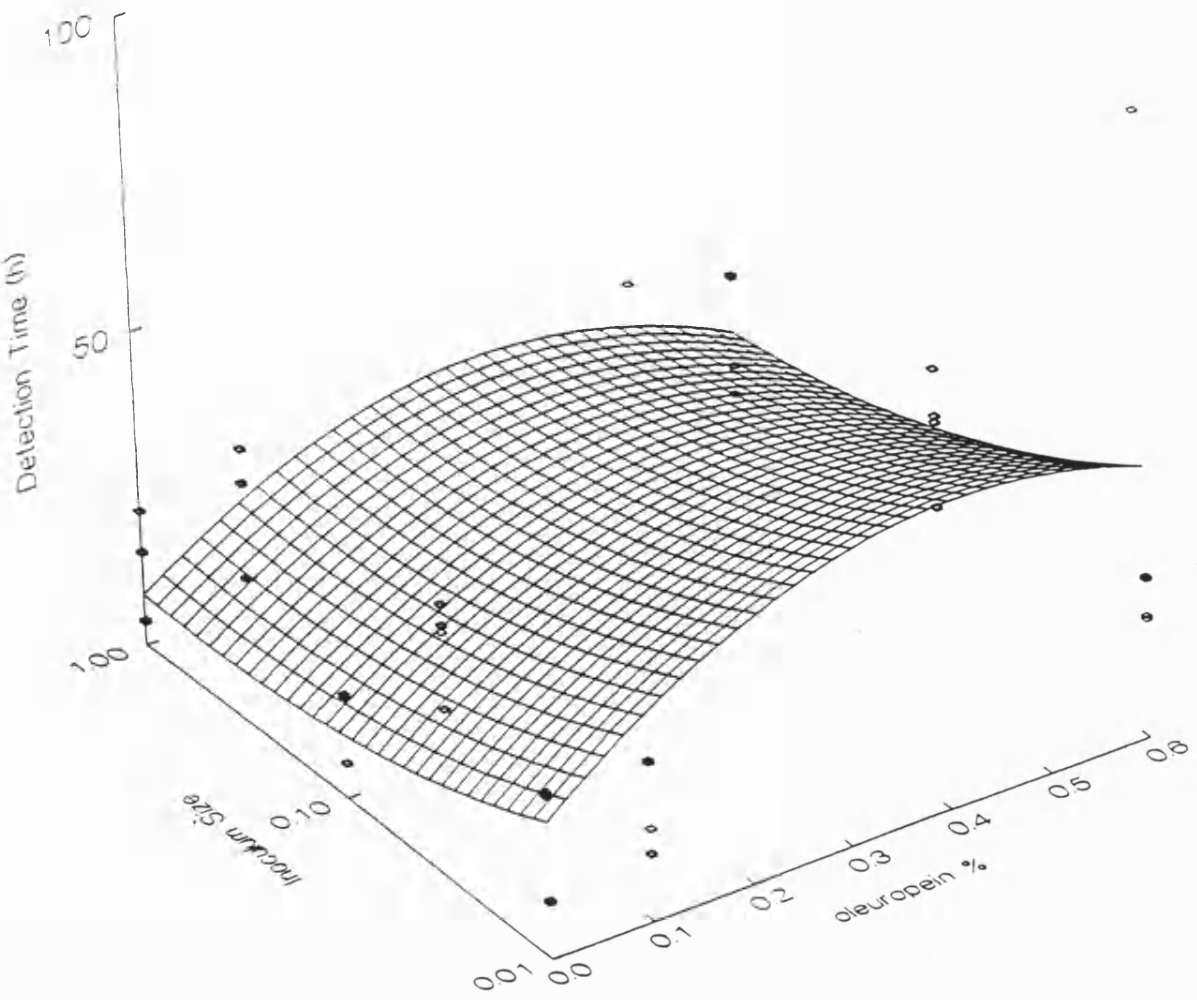


Fig. 3.20. The effect of pH and commercial oleuropein concentration (% w/v) on the detection time of *Staphylococcus aureus* S-6 in a Malthus Instrument

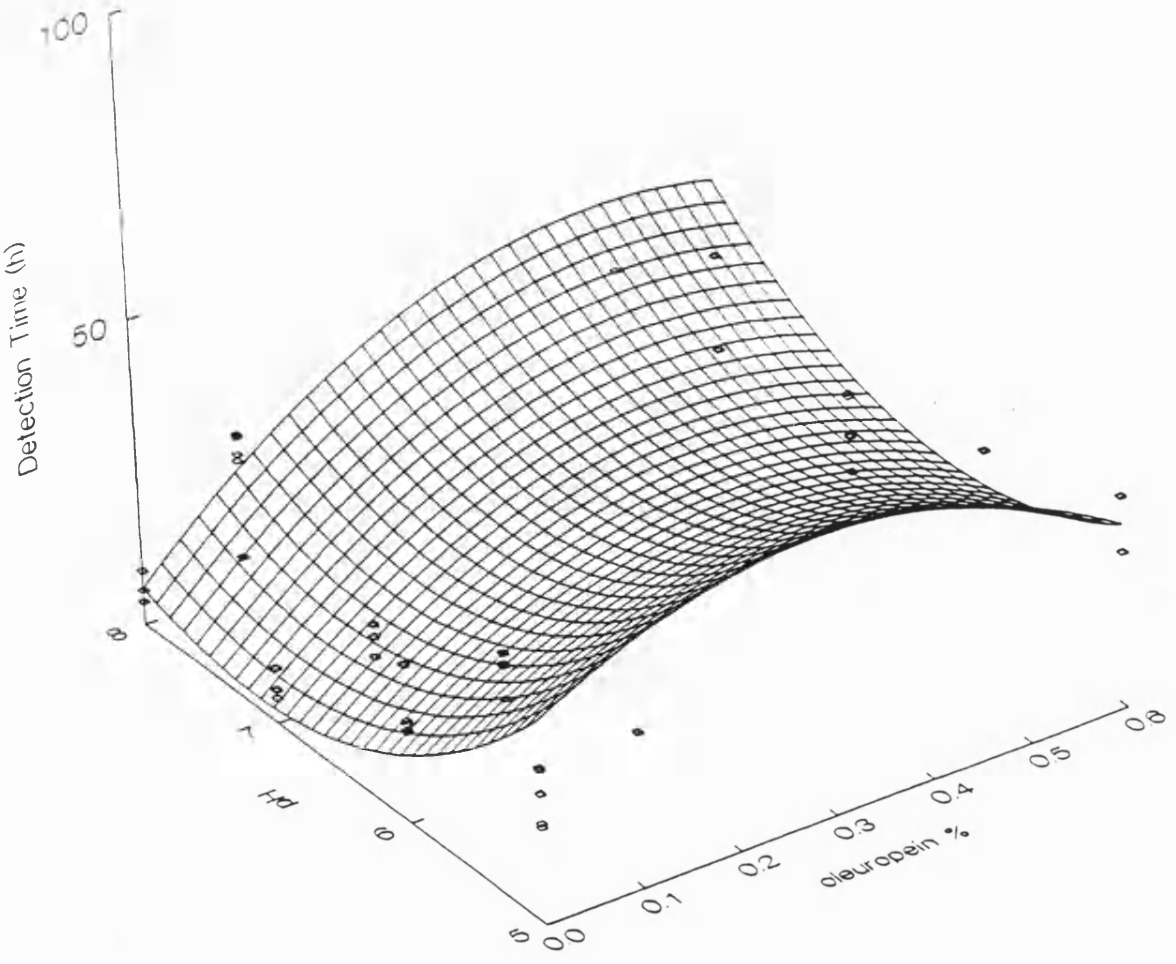
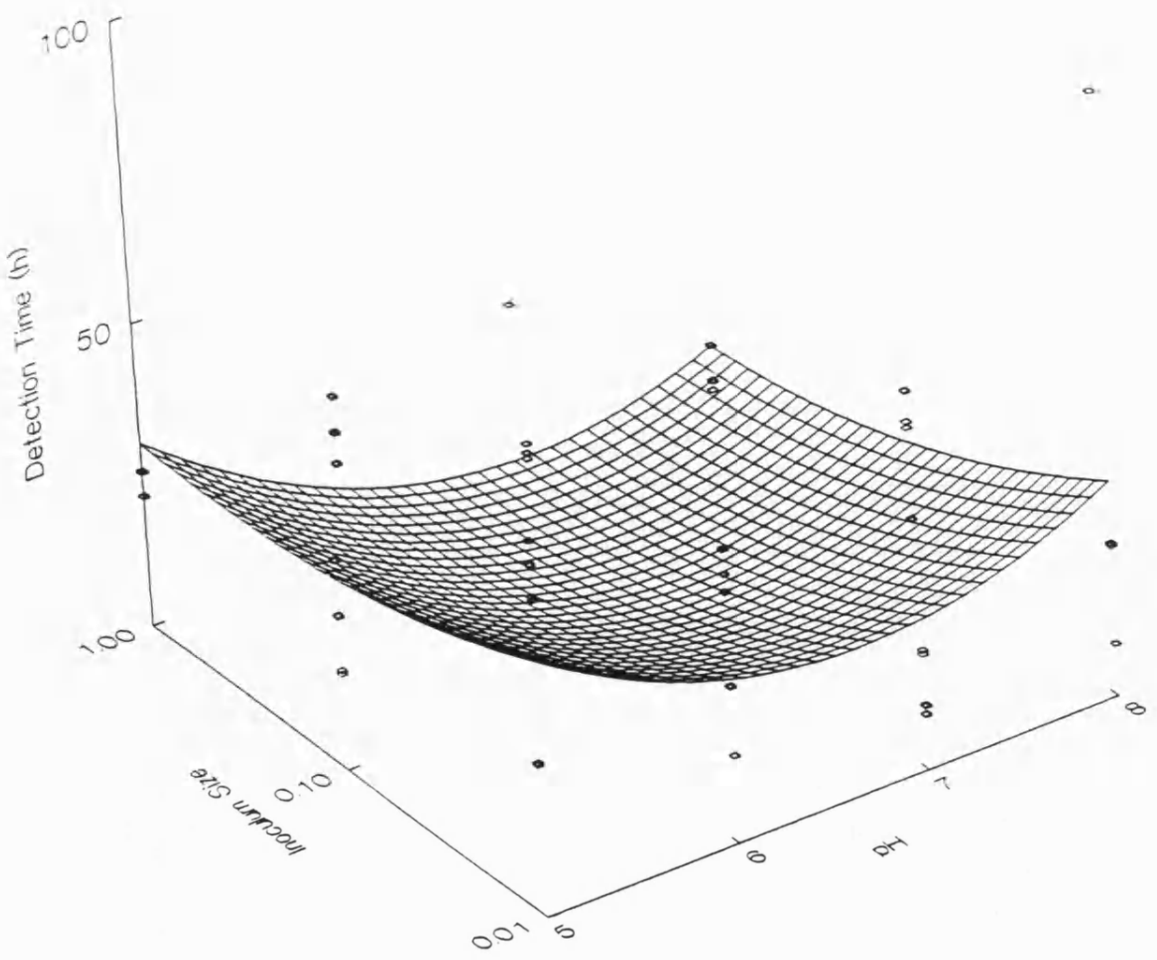


Fig. 3.21. The effect of pH and inoculum size on the detection time of *Staphylococcus aureus* S-6 in a Malthus Instrument (Inoculum size 1.00: $\log_{10}$7.15/ml; 0.10: $\log_{10}$6.15/ml; 0.01: $\log_{10}$5.15/ml)



NZA and BHI media is shown in Table 3.5. Despite the apparent difference in the increase in conductance of NZA compared to BHI in the absence of oleuropein (Figs 3.12 & 3.13), the viable counts at 48h were similar in both media. Furthermore the amount of toxin produced during the growth of *Staph. aureus* was identical in both media. With NZA, an increase in the amount of oleuropein added to the medium led to an increased detection time but a disproportionate decrease in the final (at 48h) viable count and toxin production. A concentration of 0.2% (w/v) oleuropein was sufficient to reduce the viable count to half that in the culture without oleuropein. It reduced also by 320-fold the amount of toxin produced. Indeed the low concentration of toxin measured in cultures with $>=0.2\%$ (w/v) oleuropein may represent toxin carried over with the initial inoculum although this was not verified in this experiment.

Although a similar pattern was obtained when *Staph. aureus* S-6 was inoculated into BHI (Table 3.5), 0.1% (w/v) oleuropein appeared to increase the final viable count slightly and did not have any demonstrable effect on toxin production. When compared to a control culture, this concentration of oleuropein resulted in a shorter detection time (1.5h; Table 3.5) but when the full data are shown (Fig 3.13) it can be seen clearly that the initial rapid phase of change in conductance does not begin until 8-9h after inoculation.

Although the level of toxin estimated in BHI containing $>0.2\%$ (w/v) concentrations of oleuropein appears to be ten-fold larger than that in NZA, concentrations of approximately 1ug may well be carried over with the initial inoculum and net production of toxin in both growth media is not thought to occur in the presence of concentrations of oleuropein of $>0.2\%$. The addition of oleuropein after 6h incubation, in other words at the midway point in the initial growth phase, did not reduce significantly toxin production in either medium as shown in Table 3.7.

Effect on glucose utilisation and L-lactate formation

The initial concentration (prior to addition of filter-sterilised glucose to give a final concentration *ca* 1% w/v) of glucose in both media was 31.5mg and 153.5mg in 100ml of NZA and BHI respectively (Table 3.5). The initial amount of L-lactate was found to be 0.21mg/100ml in NZA and 0.15mg/100ml in BHI.

Glucose utilisation and L-lactate formation were greater in unsupplemented NZA and BHI than in media containing oleuropein (Table 3.5). It is evident from Table 3.5 that in NZA with high concentrations of oleuropein (>=0.4% w/v) the L-lactate concentration was reduced. Such reduction was not observed in BHI containing the same concentrations of oleuropein.

Effect on the exoprotein secretion from bacteria

The addition of extract of green olives to a medium accentuated the secretion of protein by *Staph. aureus* S-6 and *Ent. faecalis* in NZA, BHI and TSB medium (Tables 3.4, 3.6, 3.9). It was notable, however, that olive extract did not affect protein secretion by *Staph.aureus* 100 in NZA or BHI (Table 3.9). The addition of glucose to NZA depressed the extent of secretion of protein by *Staph. aureus* S-6 although the bacterial density increased (Table 3.6). With the addition of olive extract the bacterial density in NZA after 9h of incubation was less than that of the control while the formation of exoproteins was higher (Table 3.6). The olive extract and oleuropein influenced also the pattern of protein secretion by *Staph. aureus* S-6 in NZA and BHI as shown by SDS-PAGE electrophoresis (Figs. 3.22, 3.23, 3.24, 3.25) or by HPLC analysis (Figs 3.26 & 3.27). The bands and peaks observed in the control samples (Figs 3.22c, 3.23a, 3.24a, 3.26b &

Table 3.9. Effect of different media and ethyl acetate extract of green olives (0.5% w/v) on exoprotein formation by *Staphylococcus aureus* S-6, *Staphylococcus aureus* 100 and *Enterococcus faecalis* at 37°C

			Microorganism		
Medium	Time (h)	Extract	*St.aureus* S-6	*St.aureus* 100	*Ent.faecalis*
	0		12.5*	12.5	12.5
NZA	9	-	92 (7.36)#	85 (6.8)	91 (7.3)
	9	+	135 (10.8)	82 (6.6)	141 (11.3)
	0		92	92	92
BHI	9	-	105 (1.14)	100 (1.08)	100 (1.08)
	9	+	160 (1.7)	98 (1.06)	125 (1.35)
	0		180	180	180
TSB	9	-	210 (1.16)	nd	200 (1.11)
	9	+	280 (1.55)		260 (1.44)

* : Mean of three samples of three replicates
\# : Times of increase of protein concentration in the substrate supernatant compared to the initial (eg 92:12.5=7.36)

Fig. 3.22. Electrophoretic patterns of culture supernatant fluids during growth of *Staphylococcus aureus* S-6: a) uninoculated NZA medium, b) addition of green olive extract after 11h of incubation and c) without olive extract after 11h of incubation

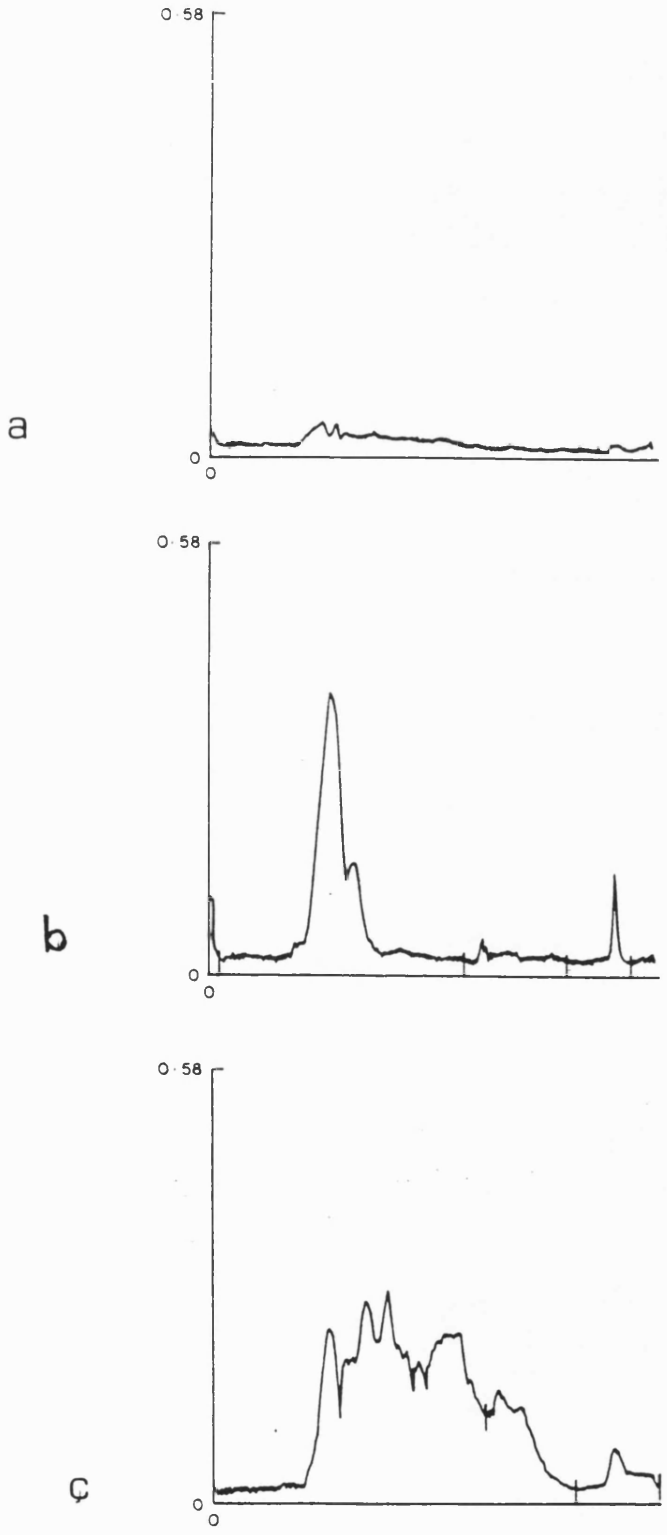


Fig. 3.23. Electrophoretic patterns of culture supernatant fluids during growth of *Staphylococcus aureus* S-6: a) in NZA medium without addition of oleuropein, b, c and d) in NZA with the addition of 0.1, 0.4 and 0.6% (w/v) oleuropein respectively

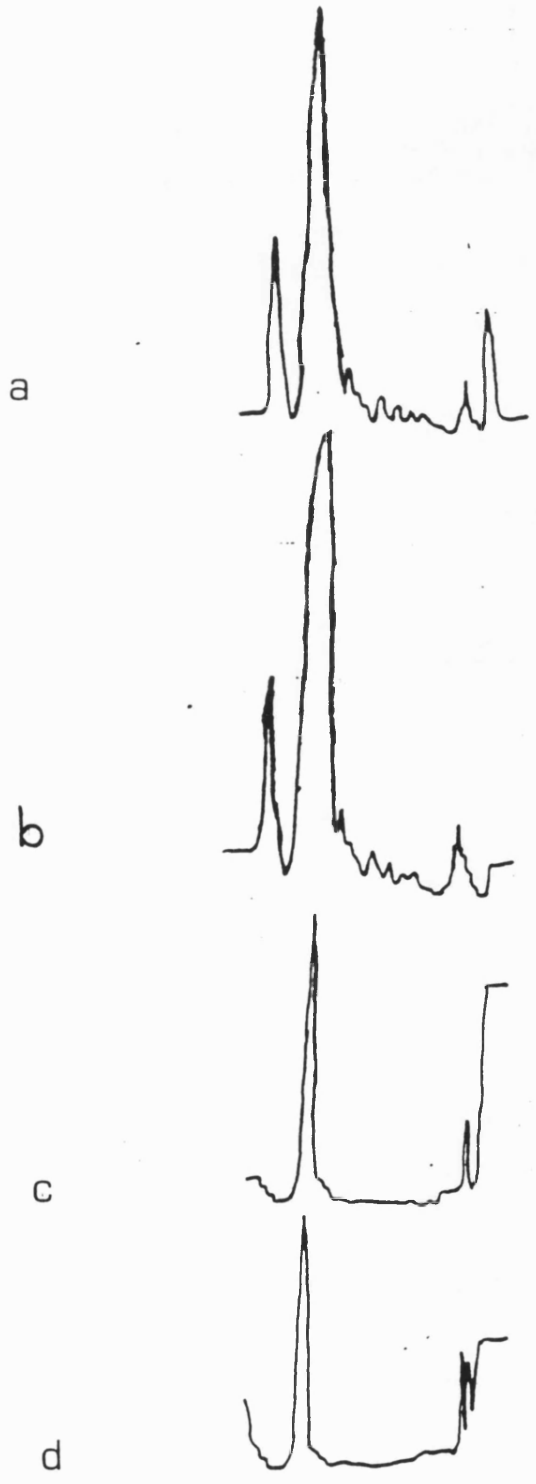


Fig. 3.24. Electrophoretic patterns of culture supernatant fluids during growth of *Staphylococcus aureus* S-6: a) in BHI medium without addition of oleuropein, b, c and d) in BHI with the addition of 0.1, 0.4 and 0.6% (w/v) oleuropein respectively

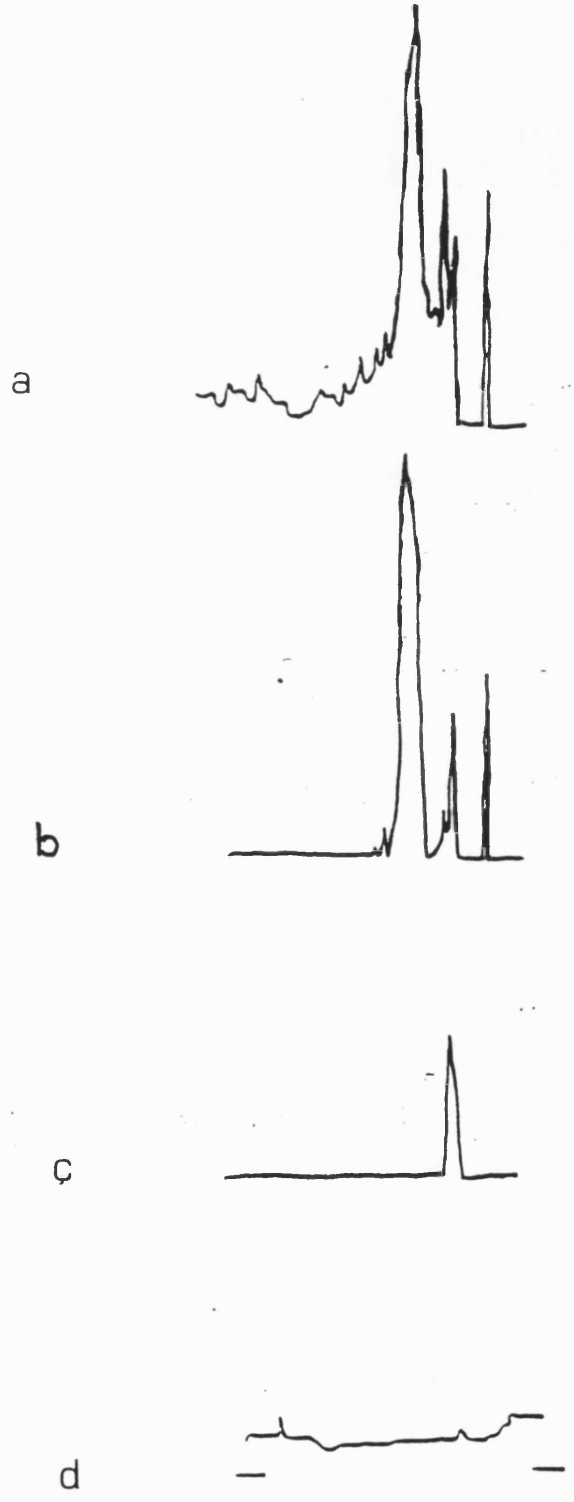
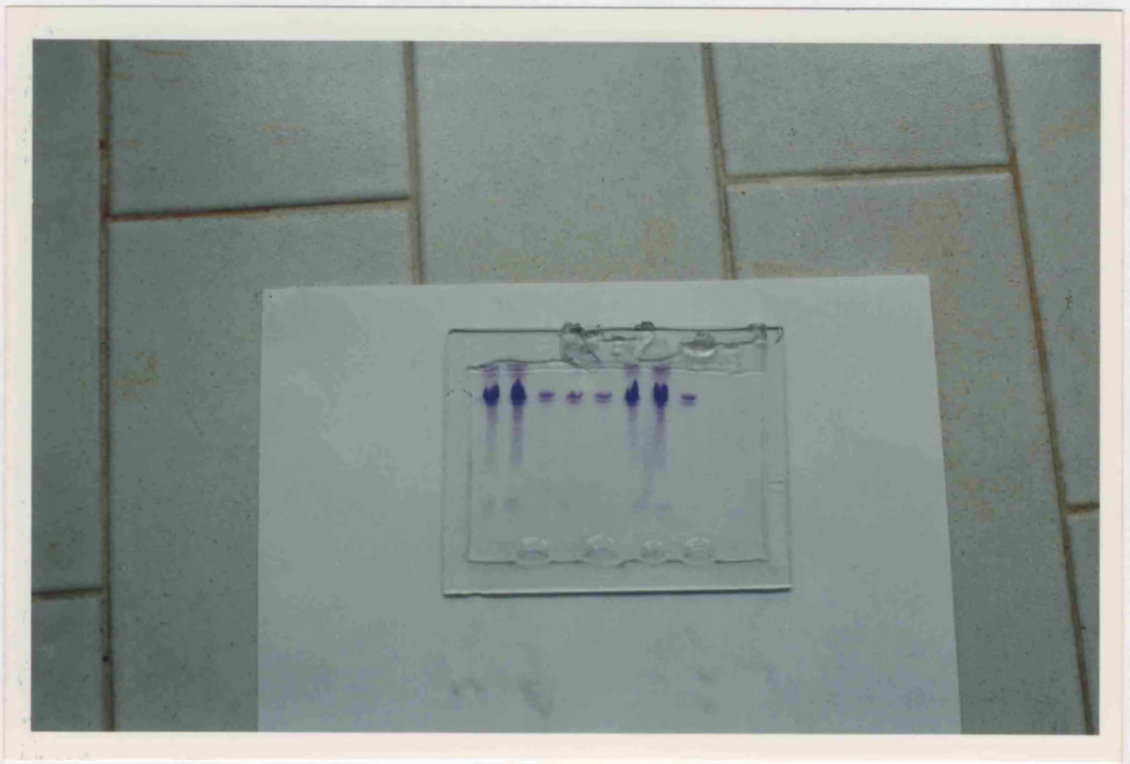


Fig. 3.25. Electrophoretic bands of samples of *Staphylococcus aureus* S-6 grown in NZA or BHI with or without the addition of oleuropein a) as shown after silver staining and b) after coomassie blue staining

a



b

Fig. 3.26. HPLC analysis of a) uninoculated NZA medium, b) NZA inoculated with *Staphylococcus aureus* S-6, c, d, e and f) inoculated NZA with addition of 0.1, 0.2, 0.4 and 0.6% (w/v) oleuropein respectively

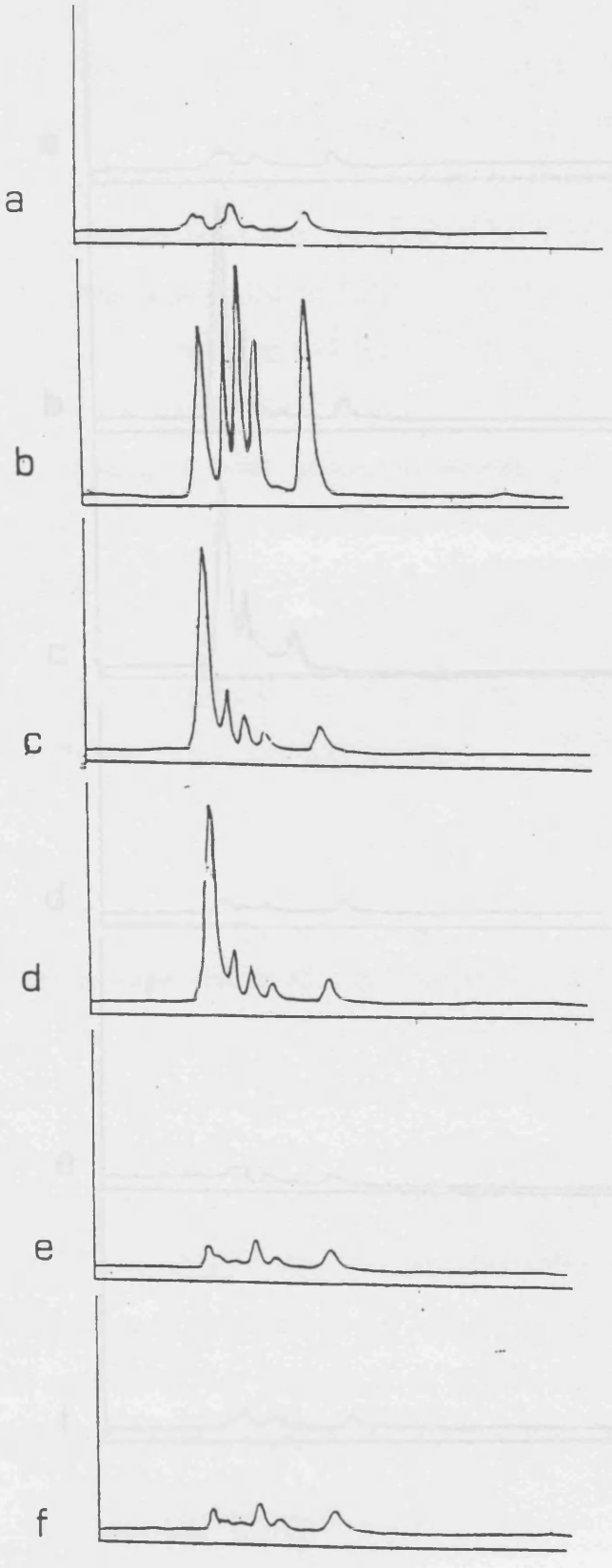
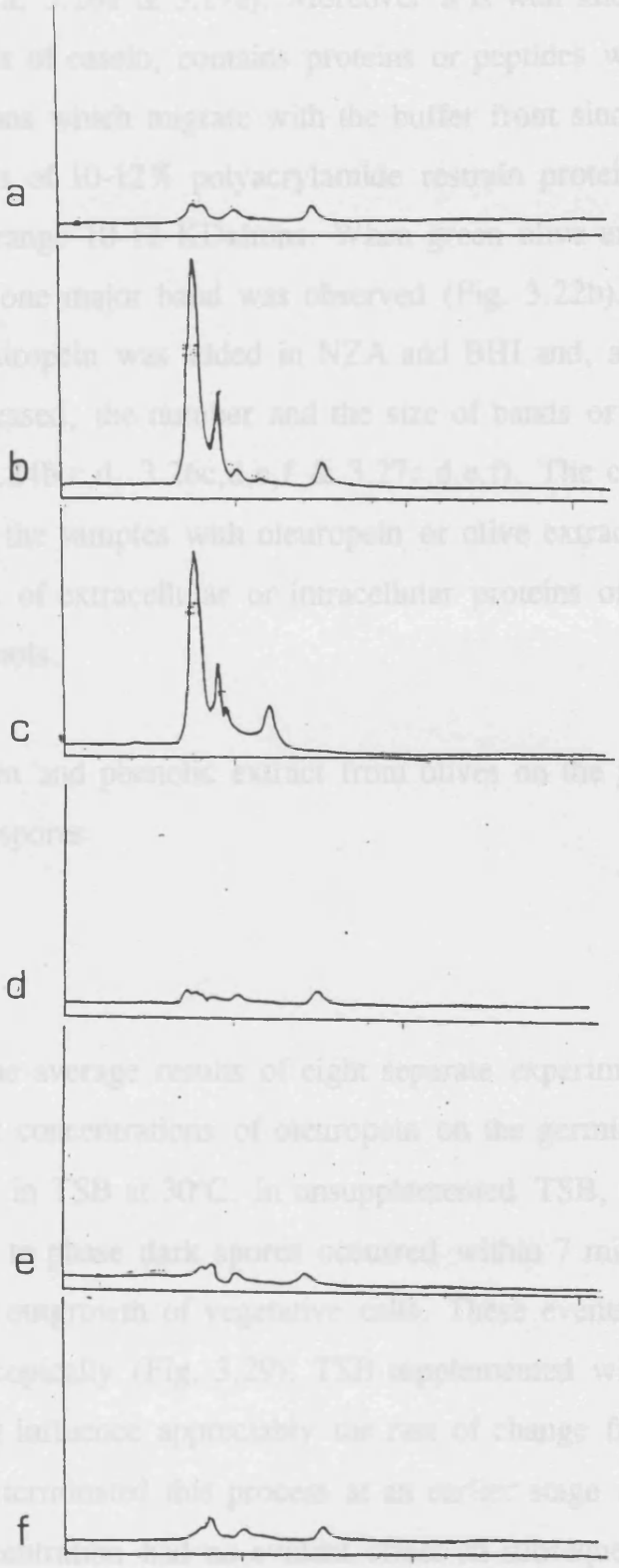


Fig. 3.27. HPLC analysis of a) uninoculated BHI medium, b) BHI inoculated with *Staphylococcus aureus* S-6, c, d, e and f) inoculated BHI with addition of 0.1, 0.2, 0.4 and 0.6% (w/v) oleuropein respectively



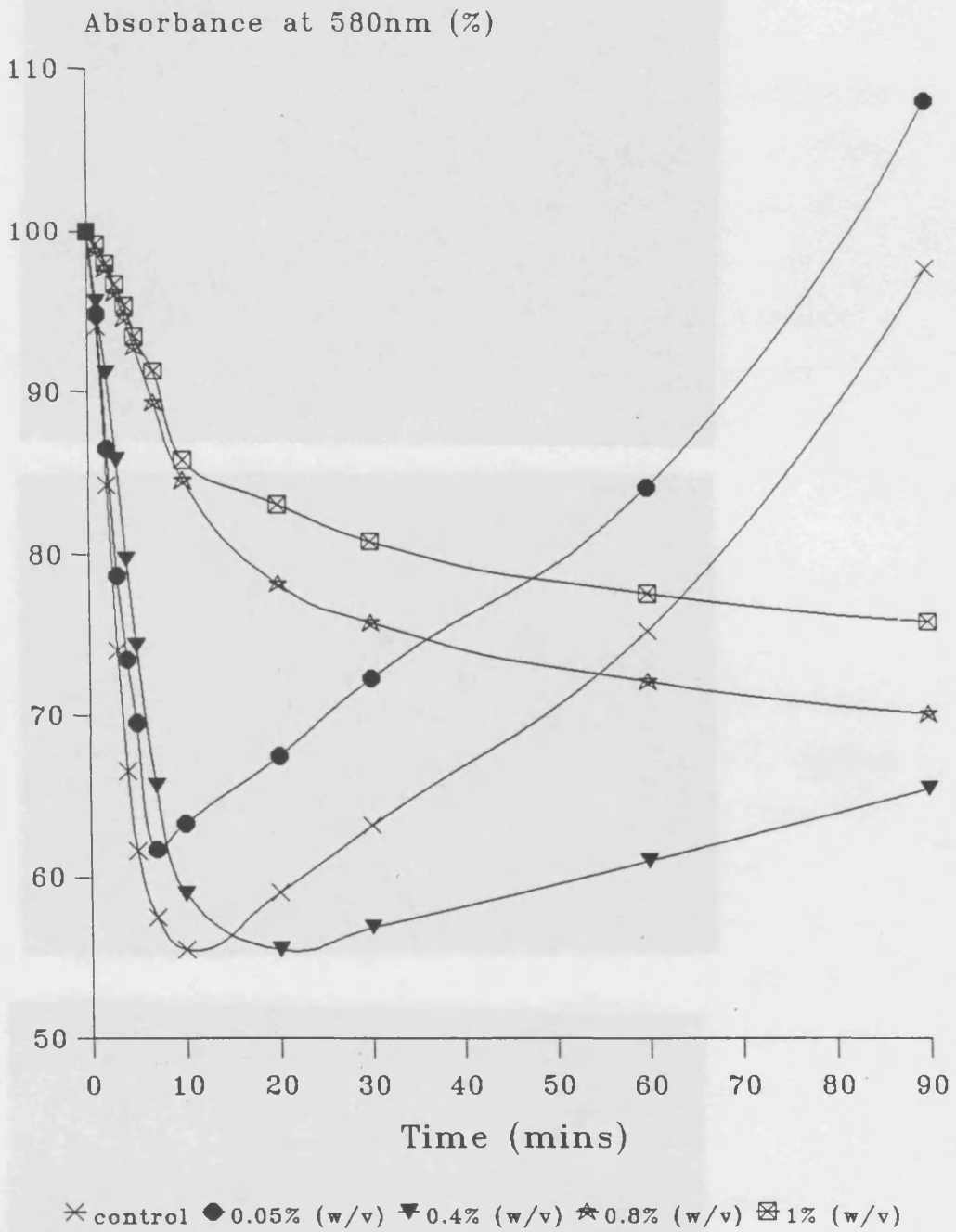
3.27b) could not be attributed to protein degradation of a medium since no detectable proteolytic activity was present in a culture supernatant fluid after 9h incubation and no bands or peaks were observed in an uninoculated medium (Figs 3.22a, 3.26a & 3.27a). Moreover it is well known that NZA, an enzymatic digest of casein, contains proteins or peptides with molecular sizes <3000 Daltons which migrate with the buffer front since electrophoresis gels of 10-12% polyacrylamide restrain proteins in the molecular weight range 10-12 KDaltons. When green olive extract was included in NZA, one major band was observed (Fig. 3.22b). Similar results obtained when oleuropein was added in NZA and BHI and, as the oleuropein concentration increased, the number and the size of bands or peaks decreased (Figs 3.23b,c,d, 3.24b,c,d, 3.26c,d,e,f & 3.27c,d,e,f). The change of the protein profiles in the samples with oleuropein or olive extract was attributed to the precipitation of extracellular or intracellular proteins of *St.aureus* caused by the phenols.

Effect of oleuropein and phenolic extract from olives on the germination of *Bacillus cereus* T spores

a. Oleuropein

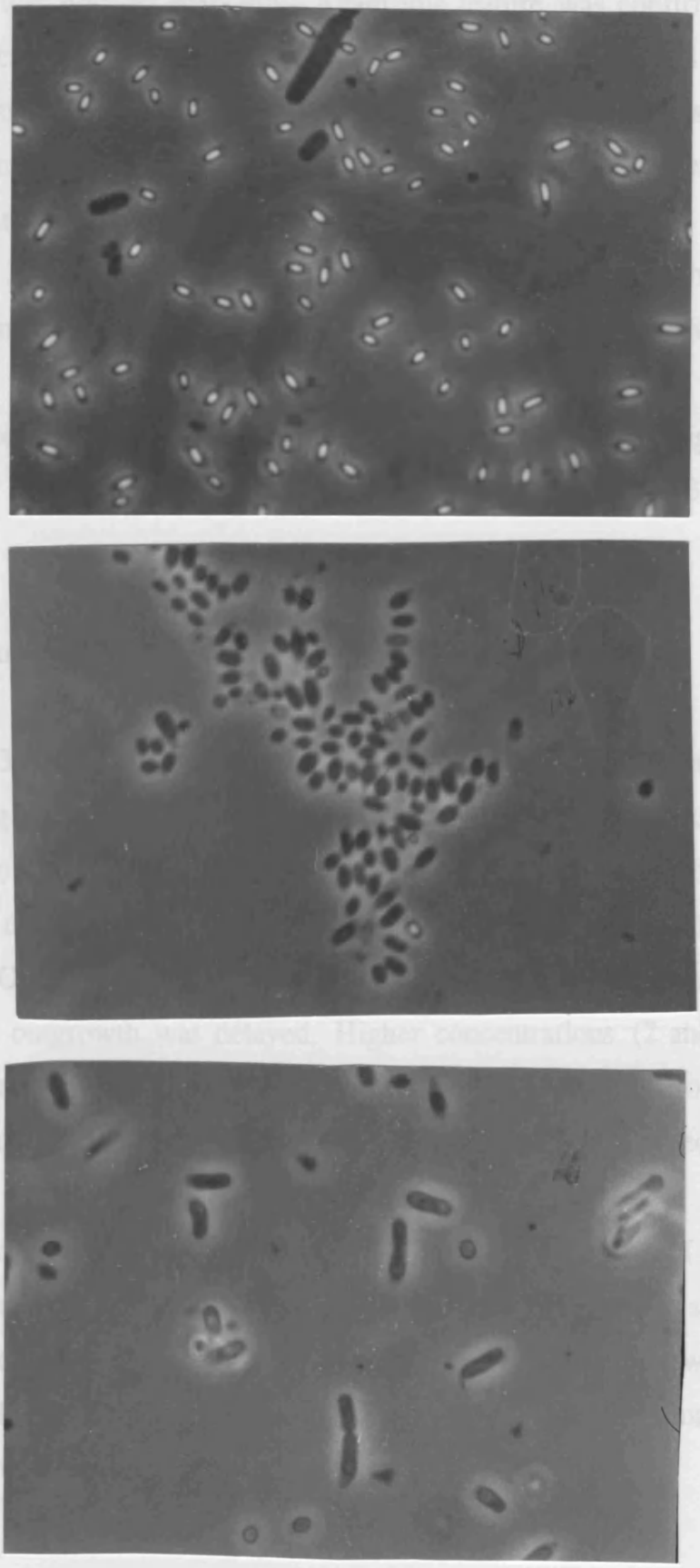
Figure 3.28 (the average results of eight separate experiments) shows the effects of different concentrations of oleuropein on the germination of *B.cereus* T spores in TSB at 30°C. In unsupplemented TSB, the changes from phase bright to phase dark spores occurred within 7 min. This was followed by rapid outgrowth of vegetative cells. These events were confirmed microscopically (Fig. 3.29). TSB supplemented with 0.05% (w/v) oleuropein did not influence appreciably the rate of change from phase bright to phase dark but terminated this process at an earlier stage *vis a vis* the control. This concentration had no evident effect on subsequent outgrowth as

Fig. 3.28. The effect of different concentrations of commercial oleuropein on spore germination and outgrowth of *Bacillus cereus* T



Each point, average of 8 experiments

Fig. 3.29. Different stages during germination process of *Bacillus cereus* T: a) phase-bright spores, b) phase-dark spores and c) outgrowth of spores and vegetative cells

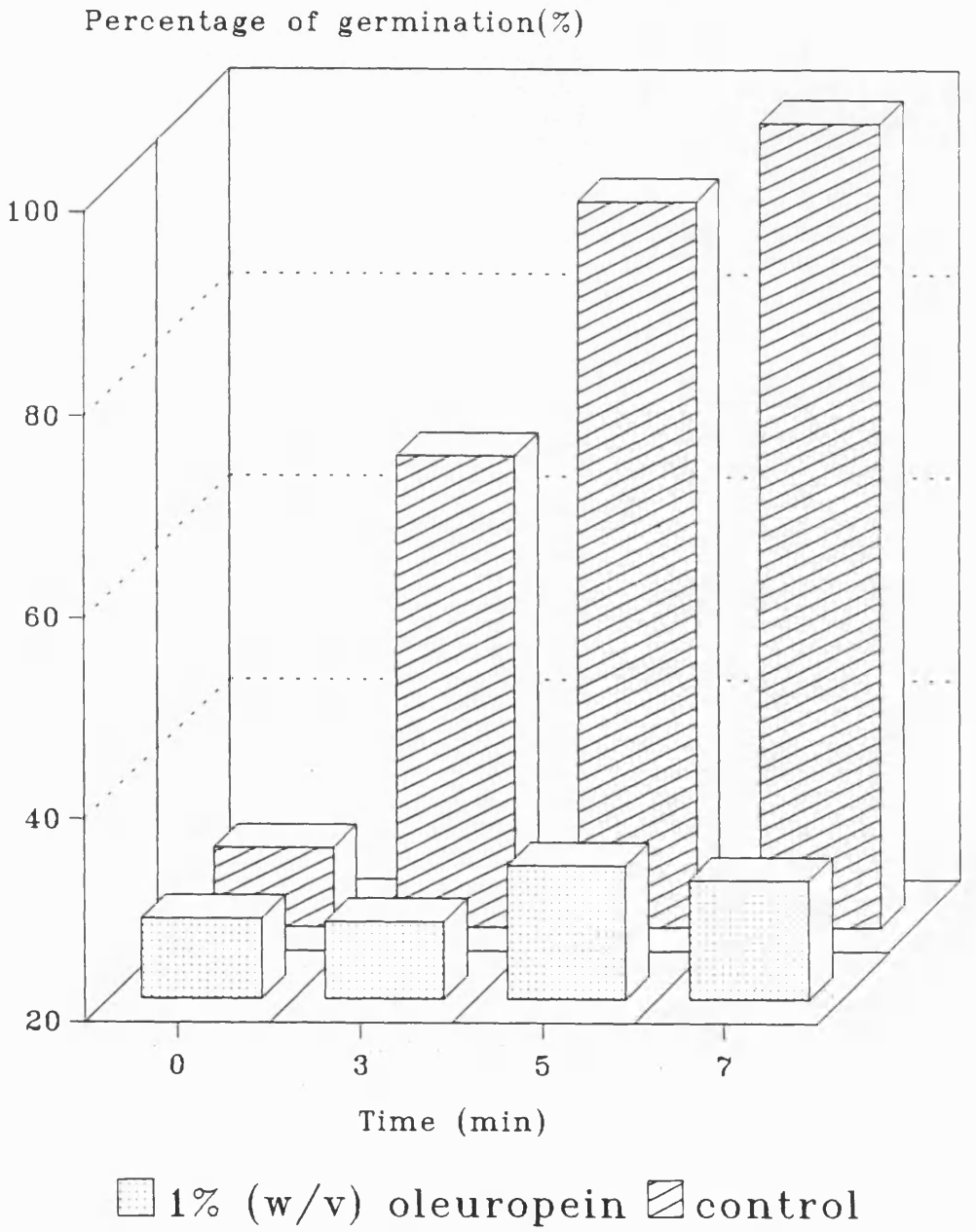


shown in microscopic studies. An oleuropein concentration of 0.4% (w/v) did not affect appreciably the phase bright to phase dark change but outgrowth was delayed markedly. Again this feature was confirmed microscopically. Higher concentrations of oleuropein [0.8 and 1%(w/v)] delayed both the rate and extent of phase change. Indeed microscopic examinations with 1% (w/v) oleuropein (Fig. 3.29 & 3.30) revealed that the vast majority of spores remained phase bright. When 1% (w/v) oleuropein was added 3 min after the initiation of germination, the rapid change of spores from phase bright to phase dark was arrested and there was no subsequent outgrowth (Fig 3.31). When the same concentration was added at 5 min, almost at the end of the phase bright to phase dark change, the germinated spores again failed to proceed to outgrowth (Fig 3.31).

b. Olive extract

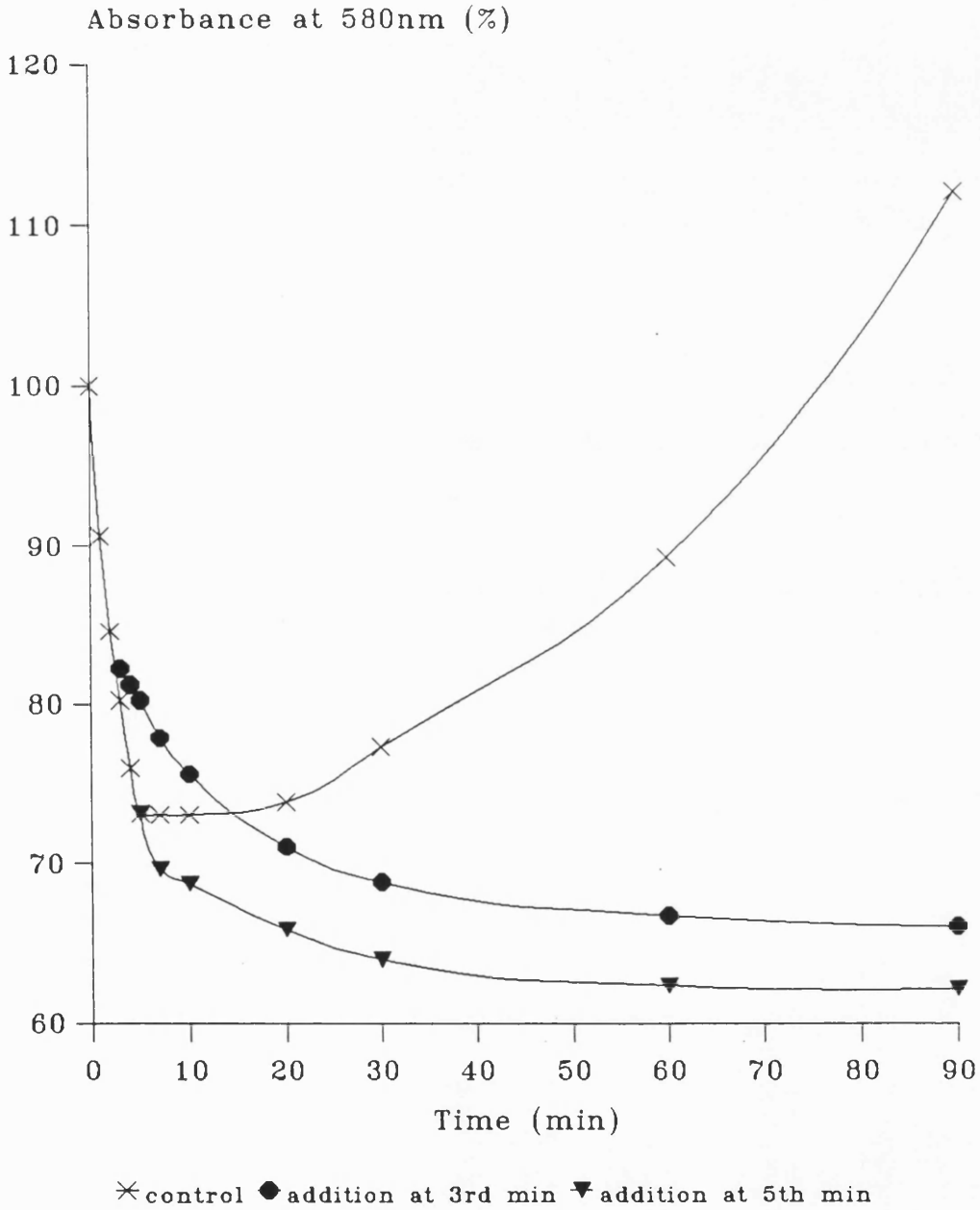
Figure 3.32 shows the effect of different concentrations of ethyl acetate extract of green olives on the germination of spores of *B. cereus* T. Addition of 1% (v/v) olive extract to the TSB spore suspension delayed both the rate and extent of change from phase bright to phase dark spores. Thus the decline in O.D. was arrested at an earlier stage than in the controls and subsequent outgrowth was delayed. Higher concentrations (2 and 4% v/v) of olive extract progressively decreased the rate of phase change of spores and inhibited completely cell outgrowth. Figure 3.33 shows the effect of 4% (v/v) olive extract when added 3 and 5 min from the beginning of germination. Addition after 3 min decreased slightly the rate of change of spores from phase bright to phase dark but delayed outgrowth significantly. The latter event was also inhibited markedly when the extract was added 5 min after the initiation of spore germination. At this time the majority of spores were phase dark.

Fig. 3.30. The effect of 1% (w/v) commercial oleuropein on the percentage of phase dark spores during germination of *Bacillus cereus* T



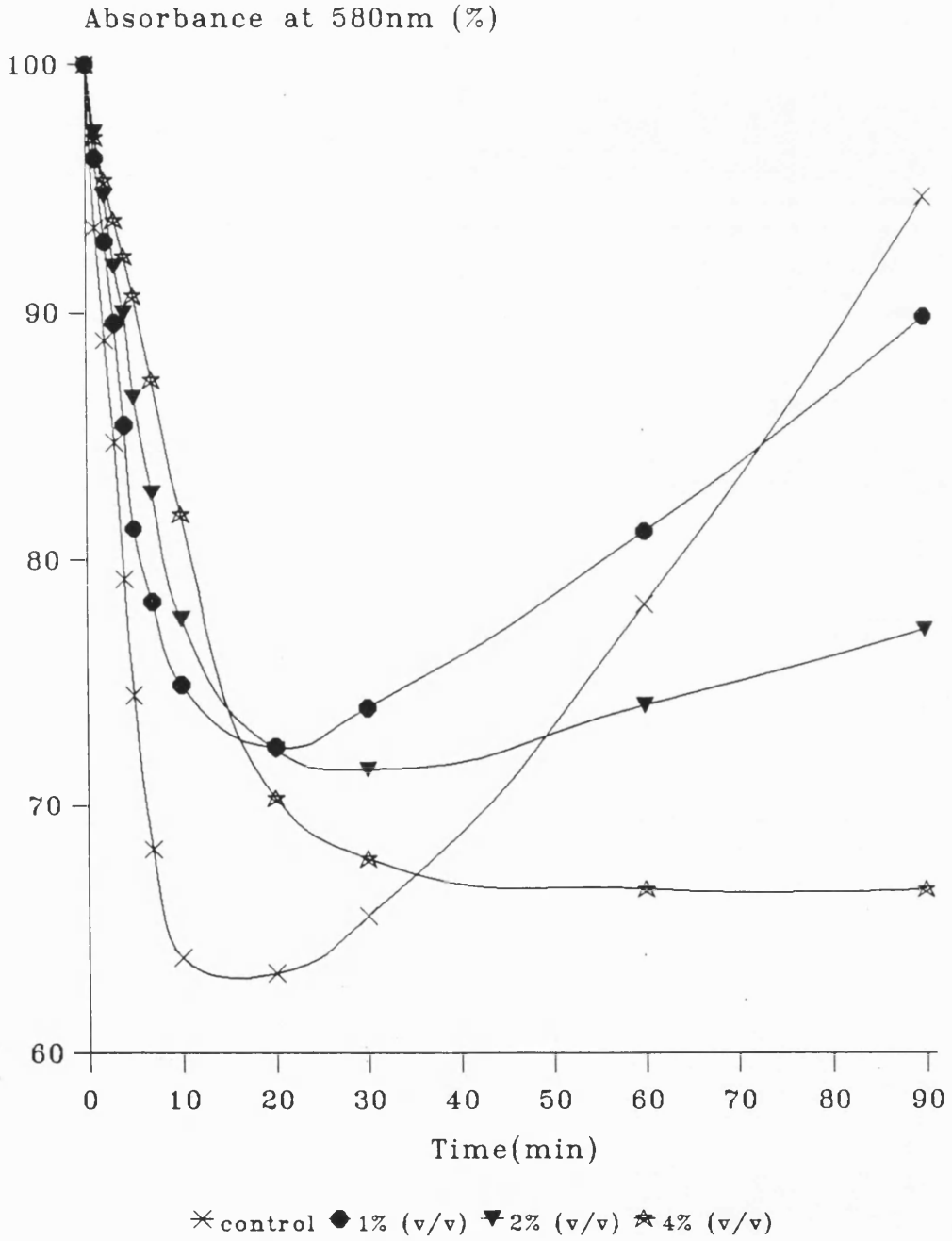
Average of 3 experiments

Fig. 3.31. The effect of 1% (w/v) commercial oleuropein added at various times of the germination process of *Bacillus cereus* T



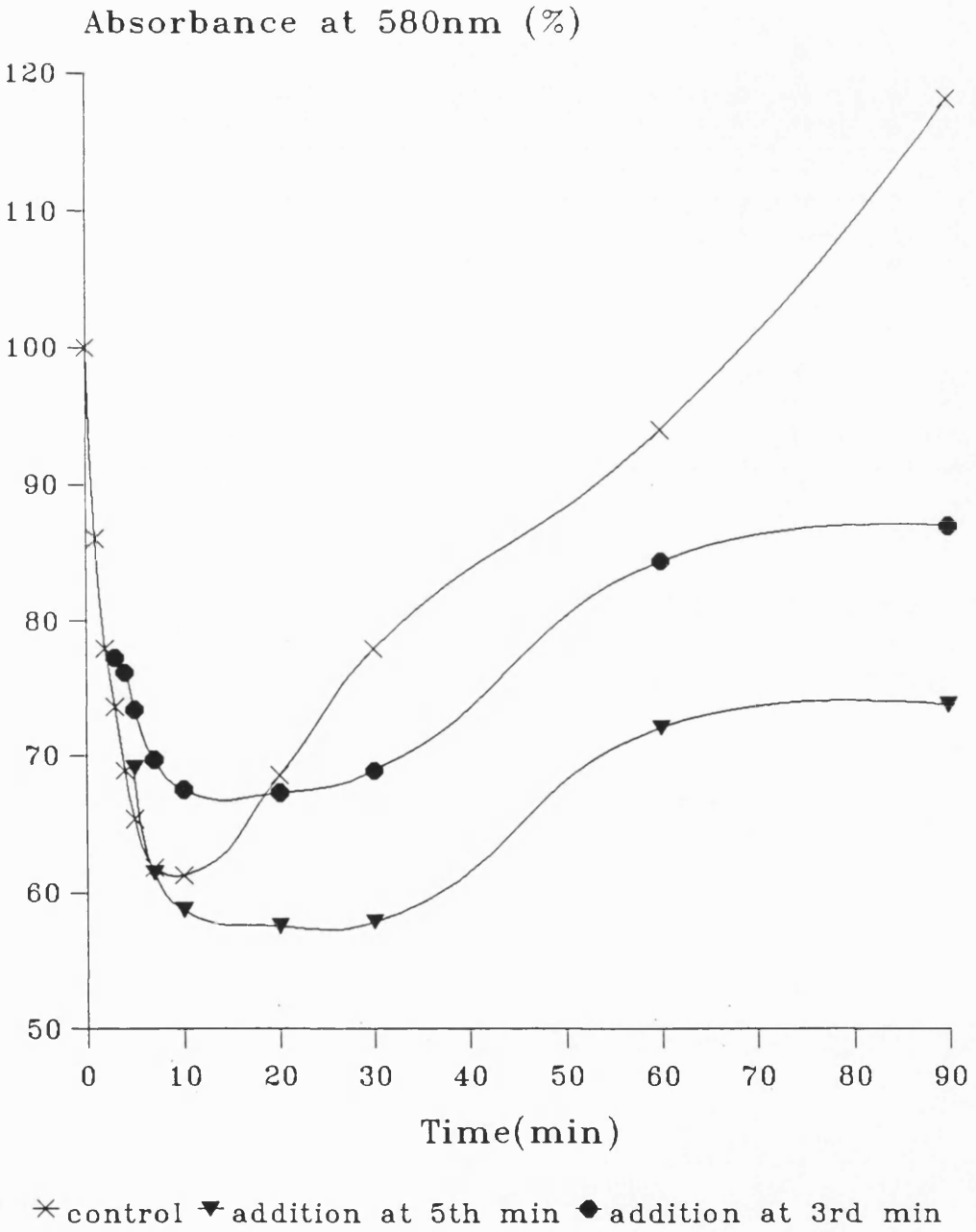
Each point average of 5 experiments

Fig. 3.32. Effect of different amounts of ethyl acetate extract of green olives on germination of *Bacillus cereus* T



Each point average of 6 experiments

Fig. 3.33. The effect of 4% (v/v) ethyl acetate extract of green olives added at various times of the germination process of *Bacillus cereus* T



Average of 2 experiments

Effect of oleuropein on the growth of *Salmonella enteritidis*

The three way analysis of variance revealed that the detection time of *Salm.enteritidis* with a Malthus Instrument was influenced by the amount of commercial (pure) oleuropein added to Coliform broth, as well as by the pH of the medium and the initial size of the inoculum (Table 3.10).

The surface analysis (3d) of these factors (% oleuropein, pH of the medium, and inoculum size) are shown in Figs 3.34, 3.35, 3.36, 3.37, 3.38, 3.39. In general the detection time was found to be longer with low pH rather than in high pH values (Fig. 3.35). Moreover the addition of oleuropein was more efficient with the lowest initial inoculum size (Fig. 3.36).

The addition of NaCl to the medium containing oleuropein did not affect significantly the growth of *Salm.enteritidis*. This is shown in Figs 3.40, 3.41 & 3.42 where the effect of 0.2% or 0.5% (w/v) of oleuropein alone or in combination with 0.5% (w/v) NaCl on three different inoculum sizes of *Salm.enteritidis* in Coliform broth. When an inoculum of $5.9x10^6$cfu/ml (Fig. 3.40) was used, 0.2% (w/v) oleuropein did not influence the increase of the conductance and the curve obtained was almost identical to that of the control. A concentration of 0.5% (w/v) oleuropein delayed by more than ten hours the detection time - in other words it extended the lag phase - but after that period the conductance increased and almost reached the values of the control. On the other hand the NaCl alone did not affect the detection time and the rate of increase in conductance but arrested the exponential phase of growth at an earlier stage than the control. A 0.2% (w/v) concentration of oleuropein in combination with NaCl (0.5% w/v) slightly delayed the detection time (1-2h) but the conductance changes were the same as those caused by the NaCl alone. When ten-times less inoculum was used ($5.9x10^5$, Fig. 3.41), the smallest concentration of oleuropein caused a longer detection time and the higher concentration did not allow any increase in the

Table 3.10. The effect of initial inoculum size, amount of commercial oleuropein added and the pH of the medium, on the detection time (a) and on the final growth (b) of *Salmonella enteritidis* in Coliform broth at 37°C (Three way analysis of variance)

a

Source	df	f-ratio	Probability
(A) Inoculum size	2	8755	0.0000
(B) Oleuropein	3	797	0.0000
(C) pH	2	3268	0.0000
A x B	6	238	0.0000
A x C	4	435	0.0000
B x C	6	789	0.0000
A x B x C	12	170	0.0000

b

Source	df	f-ratio	Probability
(A) Inoculum size	2	43643	0.0000
(B) Oleuropein	2	28978	0.0000
(C) pH	3	35314	0.0000
A x B	4	4501	0.0000
A x C	6	9514	0.0000
B x C	6	17349	0.0000
A x B x C	12	15035	0.0000

Inoculum size : $\log_{10}7.3$/ml, $\log_{10}6.3$/ml, $\log_{10}5.3$/ml
Oleuropein concentration: 0, 0.1, 0.6% (w/v)
pH : 5.5, 6, 7, 7.8

Fig. 3.34. The effect of pH and inoculum size on the detection time of *Salmonella enteritidis* in Coliform broth at 37°C as determined with a Malthus Instrument (Inoculum size 1.00: log_{10}7.3/ml; 0.10: log_{10}6.3/ml; 0.01: log_{10}5.3/ml)

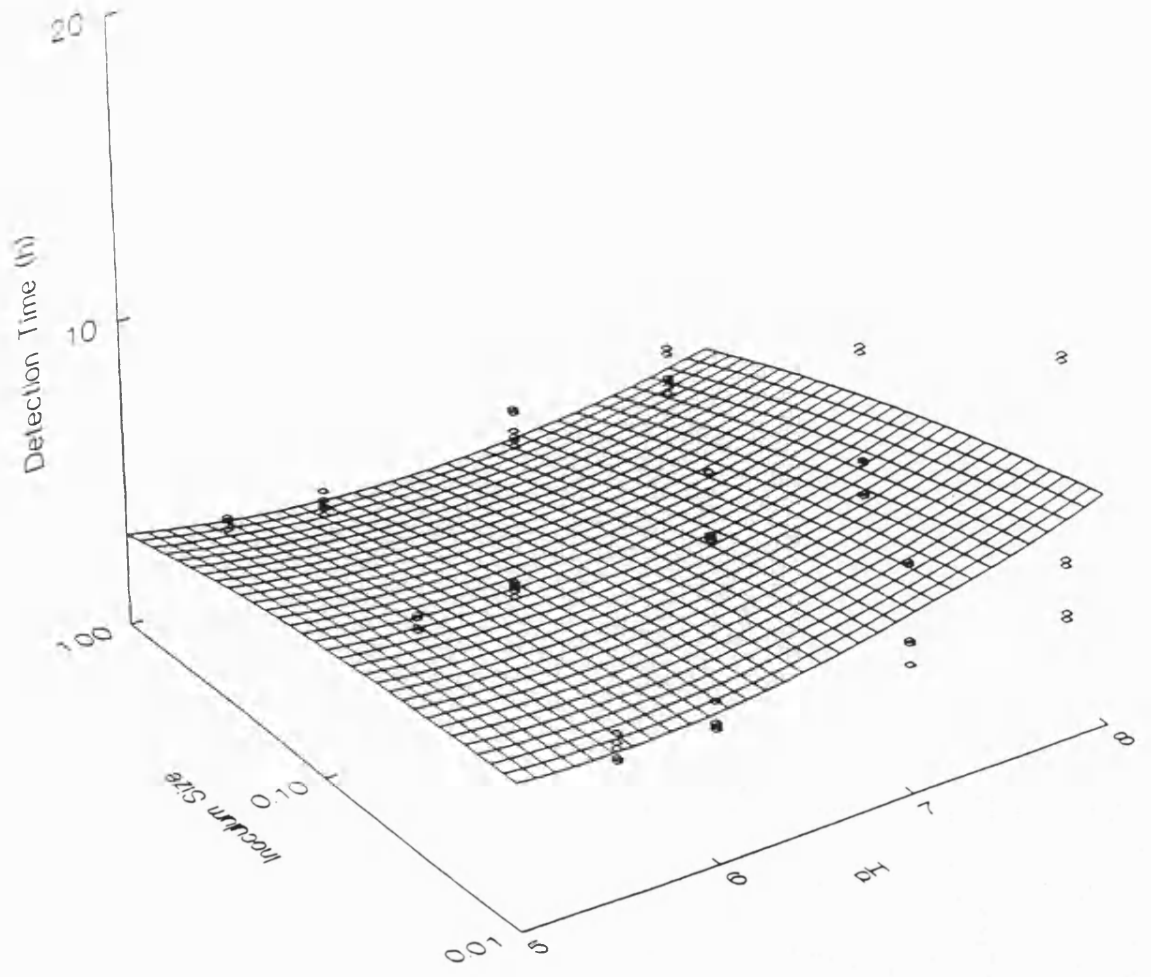


Fig. 3.35. The effect of pH and commercial oleuropein concentration (% w/v) on the detection time of *Salmonella enteritidis* in Coliform broth at 37°C as determined with a Malthus Instrument

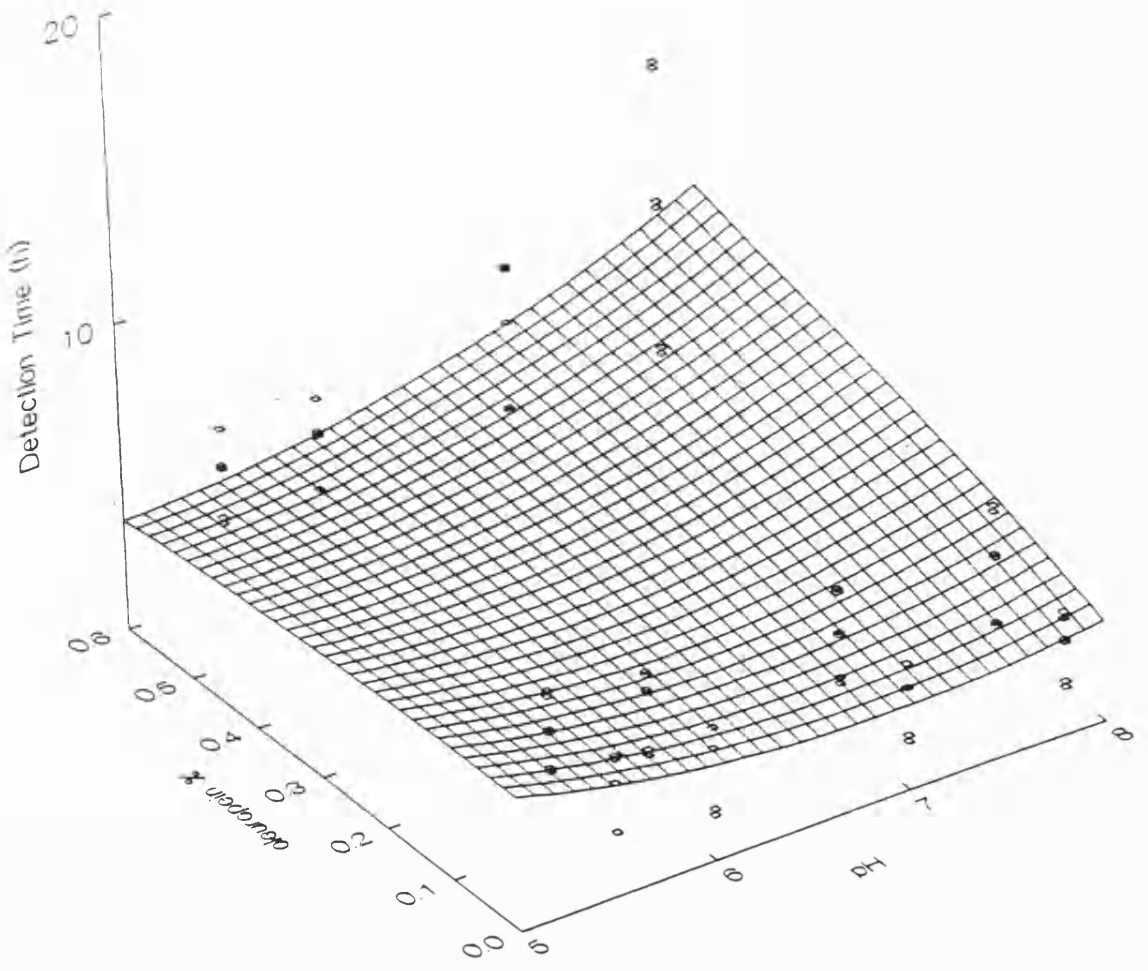


Fig. 3.36. The effect of commercial oleuropein concentration (% w/v) and inoculum size on the detection time of *Salmonella enteritidis* in Coliform broth at 37°C as determined with a Malthus Instrument (Inoculum size 1.00: log_{10}7.3/ml; 0.10: log_{10}6.3/ml; 0.01: log_{10}5.3/ml)

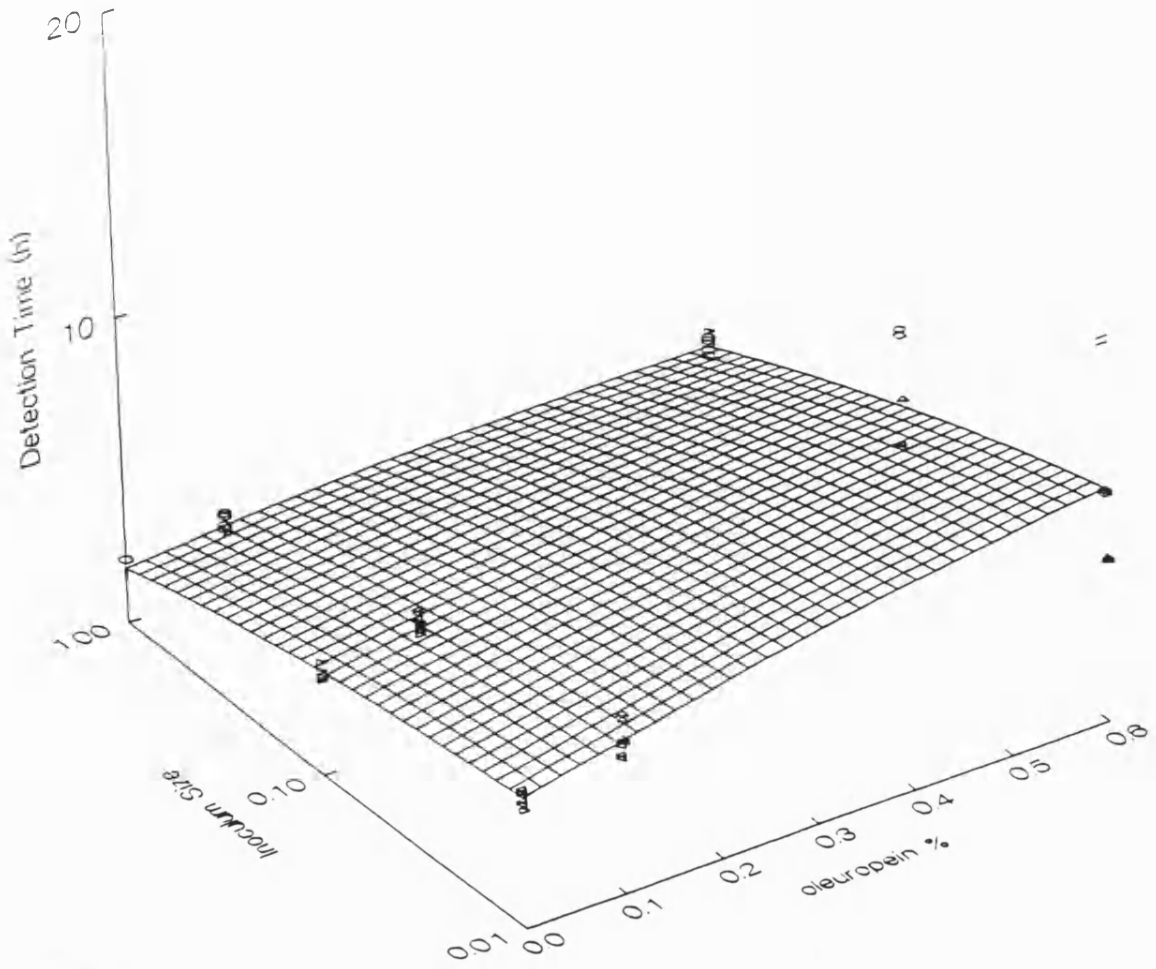


Fig. 3.37. The effect of commercial oleuropein concentration (% w/v) and pH on the final growth of *Salmonella enteritidis* in Coliform broth at 37°C as determined with a Malthus Instrument

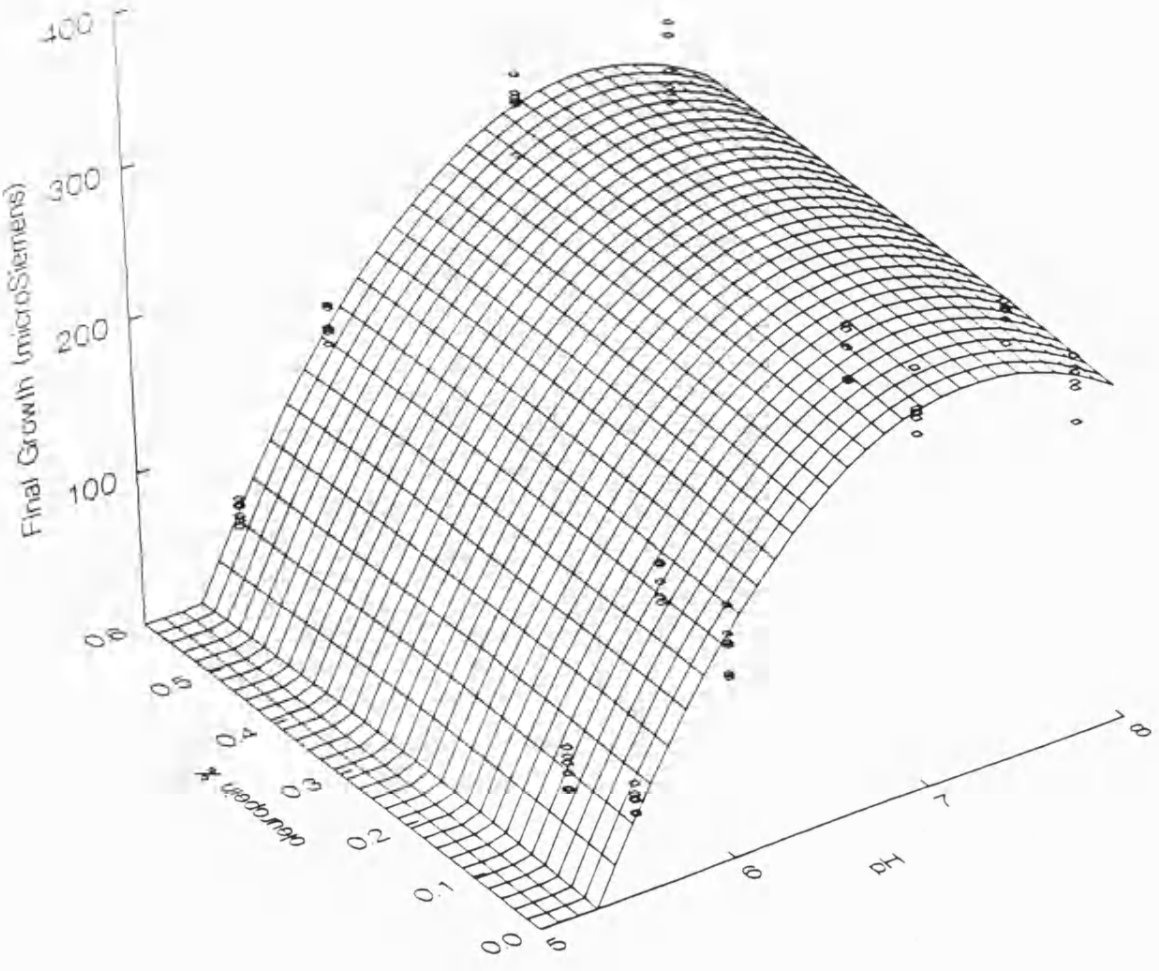


Fig. 3.38. The effect of pH and inoculum size on the final growth of *Salmonella enteritidis* in Coliform broth at 37°C as determined with a Malthus Instrument (Inoculum size 1.00: $\log_{10}$7.3/ml; 0.10: $\log_{10}$6.3/ml; 0.01: $\log_{10}$5.3/ml)

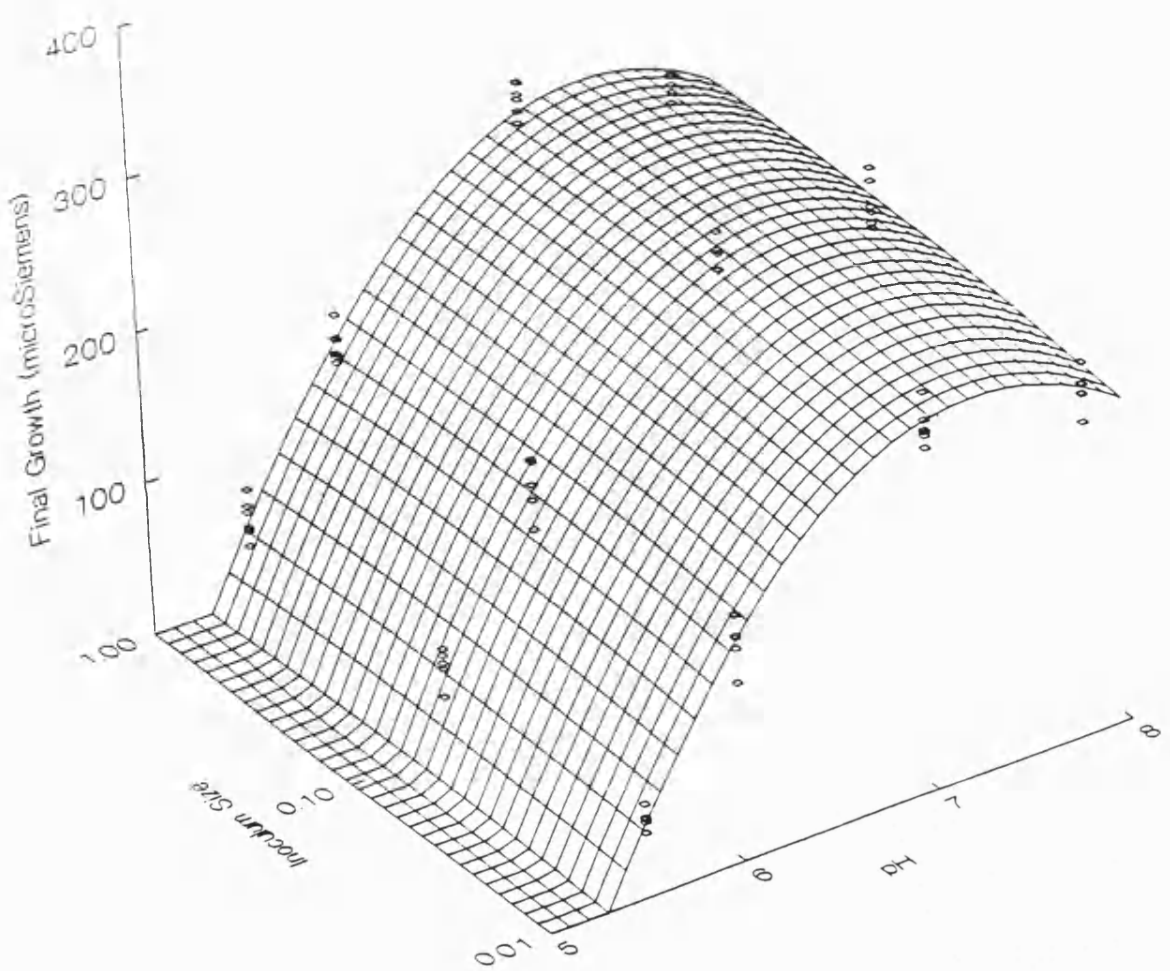


Fig. 3.39. The effect of commercial oleuropein concentration (% w/v) and inoculum size on the final growth of *Salmonella enteritidis* in Coliform broth at 37°C as determined with a Malthus Instrument (Inoculum size 1.00: $\log_{10}$7.3/ml; 0.10: $\log_{10}$6.3/ml; 0.01: $\log_{10}$5.3/ml)

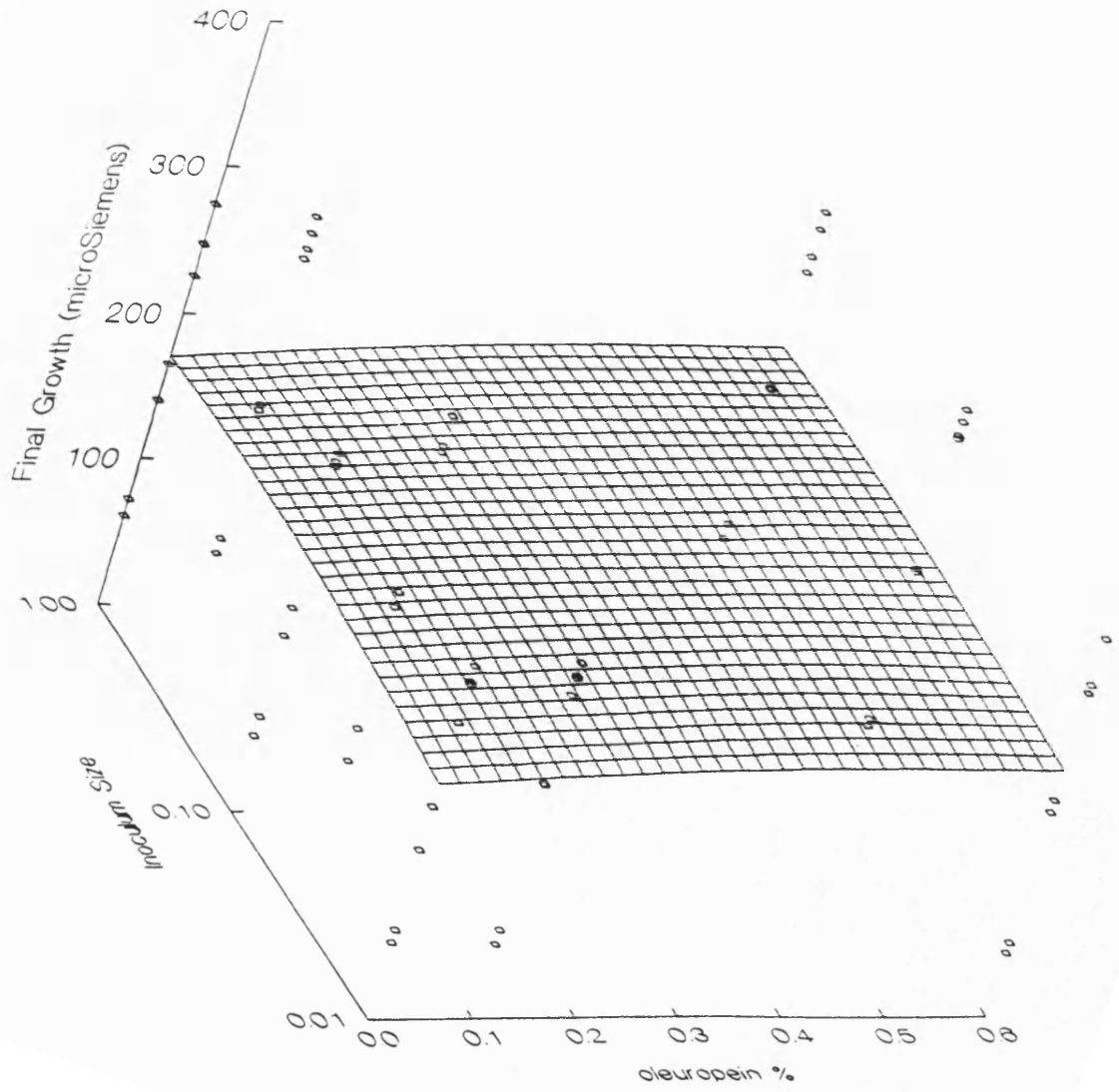
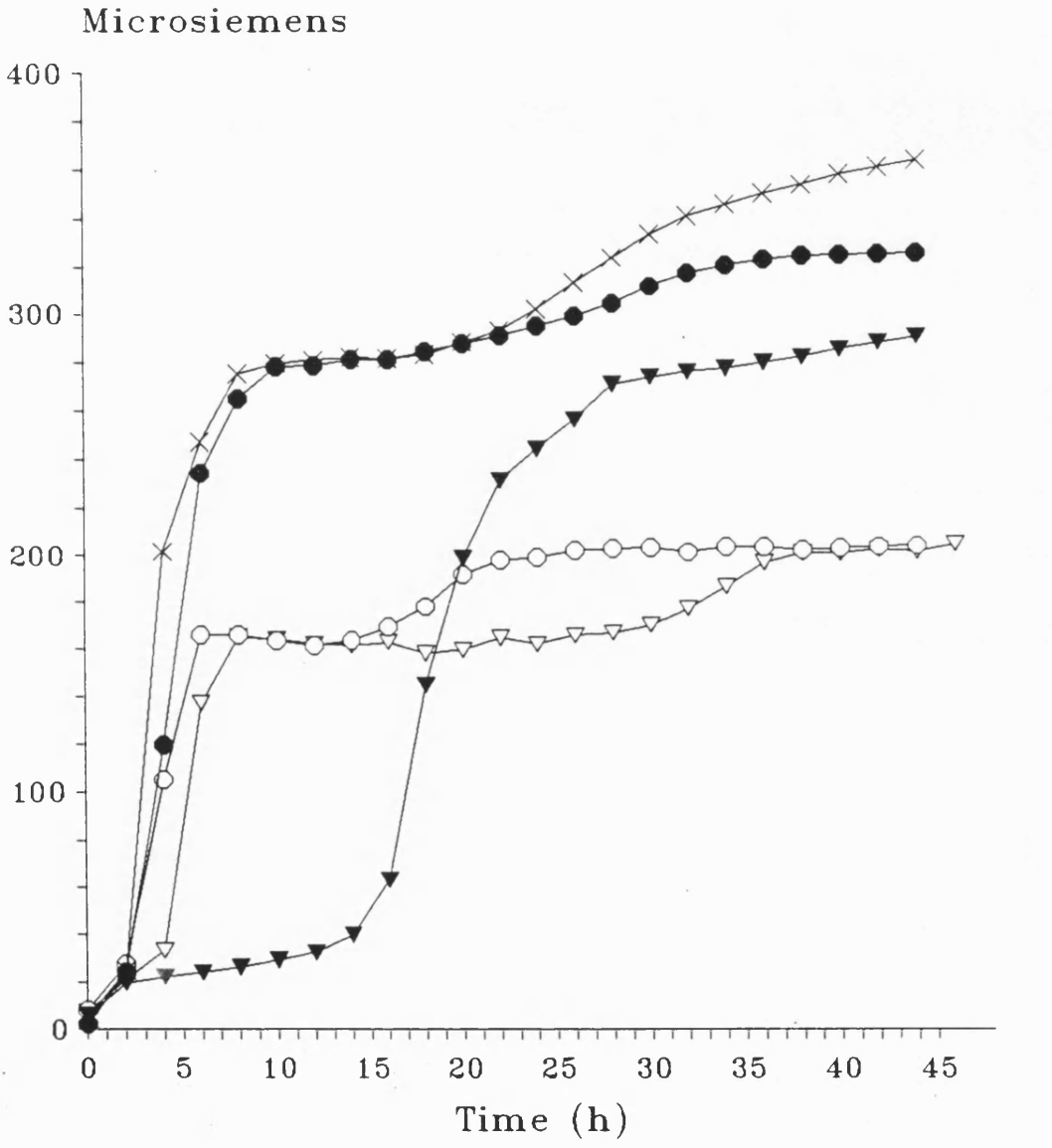
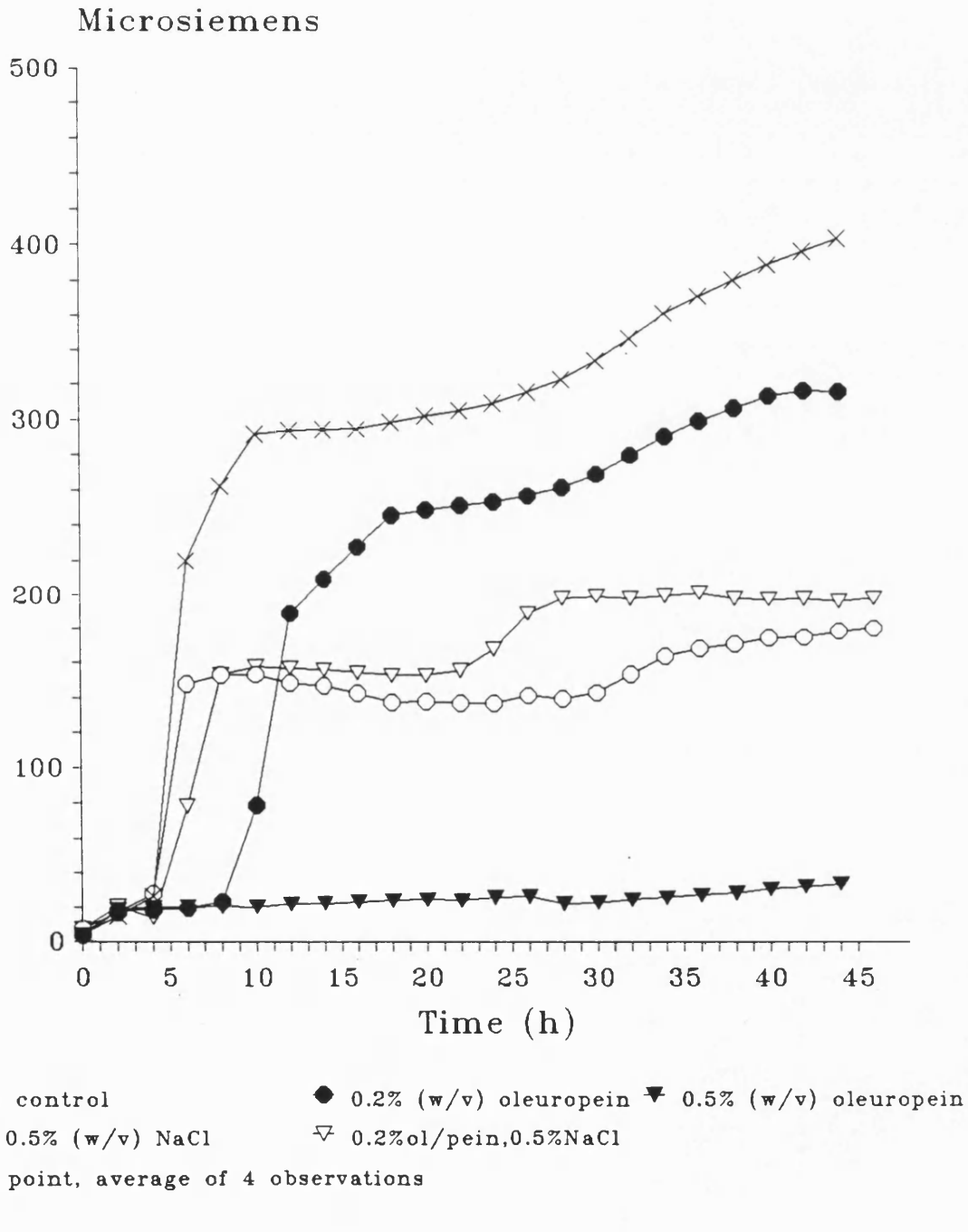


Fig. 3.40. The effect of commercial oleuropein and NaCl on the growth of *Salmonella enteritidis* (inoculum size $\log_{10}$6.8cfu/ml) in Coliform broth at 37°C in a Malthus Instrument



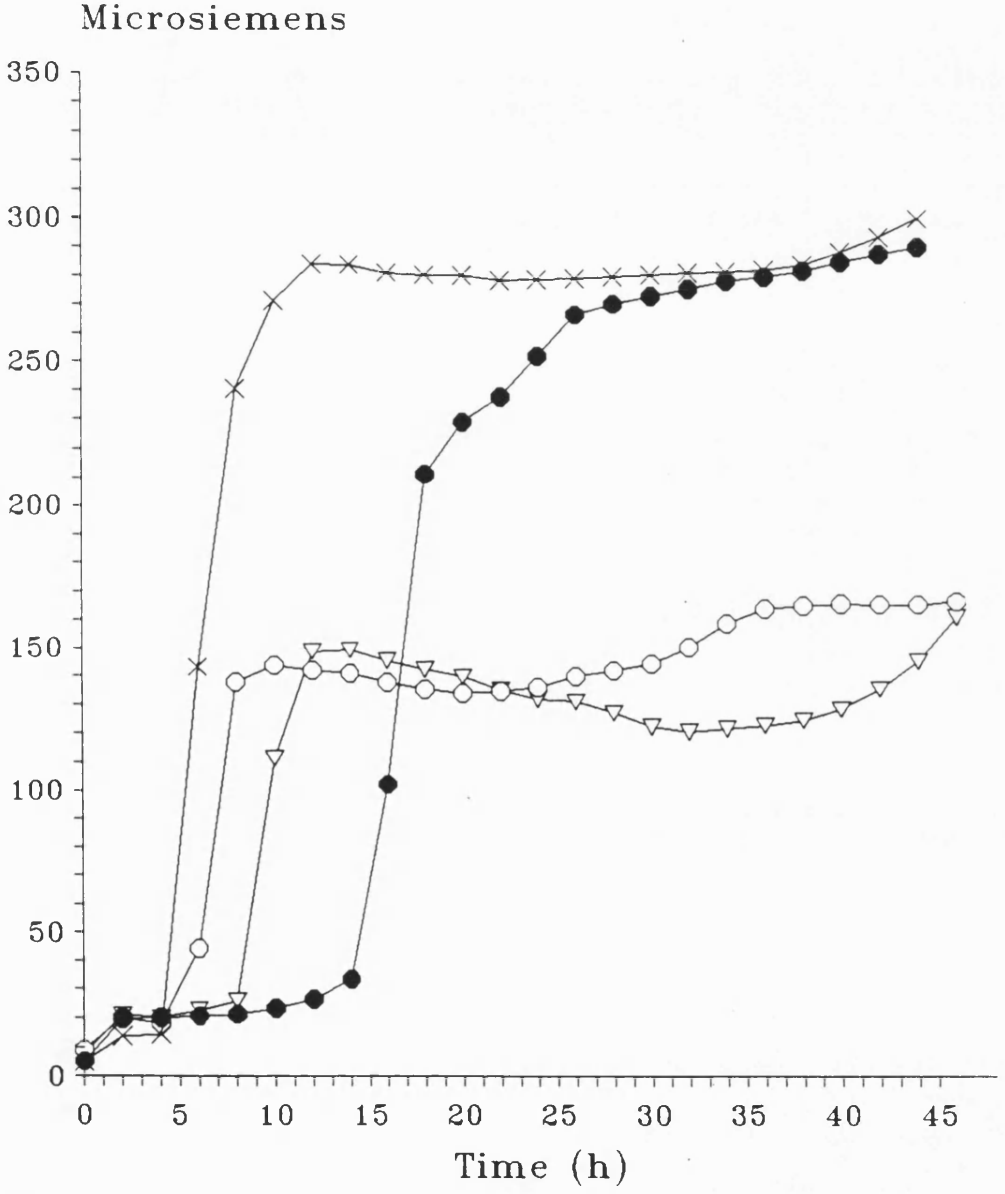
Each point, average of 4 observations

Fig. 3.41. The effect of commercial oleuropein and NaCl on the growth of *Salmonella enteritidis* (inoculum size $\log_{10}$5.8cfu/ml) in Coliform broth at 37°C in a Malthus Instrument



Each point, average of 4 observations

Fig. 3.42. The effect of commercial oleuropein and NaCl on the growth of *Salmonella enteritidis* (inoculum size $\log_{10}$4.8cfu/ml) in Coliform broth at 37°C in a Malthus Instrument



✳ control ● 0.2% (w/v) oleuropein ○ 0.5% (w/v) NaCl ▽ 0.2%ol/pein,0.5%NaCl

Each point, average of 4 observations

conductance of the Coliform broth in which salmonellas were grown. With the NaCl alone no change due to the inoculum size was observed.

The results obtained with oleuropein and NaCl were accentuated when a ten-times less inoculum was used ($5.9x10^4$, Fig. 3.42). In this case the oleuropein (0.2% w/v) alone caused an extension of the lag phase but when it was used in combination with NaCl, the effect was less.

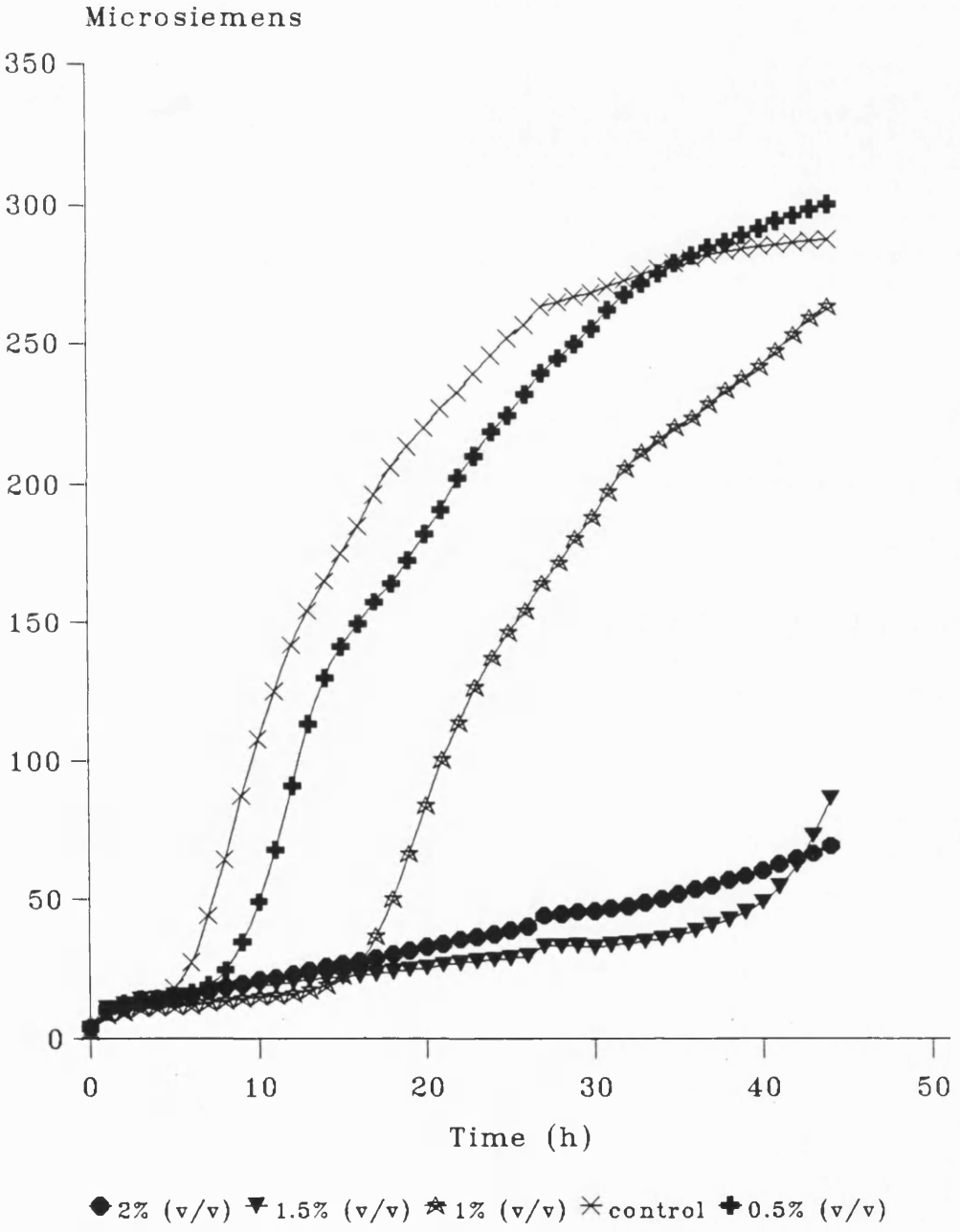
In model food system

Effect of phenolic compounds extracted from green olives (Fig. 2.9b) on the growth of *Staphylococcus aureus* S-6 in milk.

Staphylococcus aureus inoculated in sterile reconstituted milk grew extensively as it is shown in Fig 3.43. When 0.5% (v/v) of olive extract was added the detection time was delayed by almost 3 hours but there was no influence on the growth or conductance curve as obtained with the Malthus system (Table 3.11). A concentration of 1% (v/v) of extract delayed the detection time to almost 11h (Table 3.11) while 1.5 and 2% (v/v) olive extract caused a significant delay in the detection times or a reduction in the final height of the conductance curves. Although there was apparent inhibition of microbial activity with high concentrations of olive extract, no appreciable differences in the viable counts were noted (Table 3.11). Indeed the viable counts in the case of 2% (v/v) extract was decreased about 1.5 $\log_{10}$ less than that of the control. However the toxin production by the microorganism seemed to follow the curves of the microbial activity and not the viable counts since the toxin diminished to about a half of that of the control in the case of 0.5 or 1% (v/v) of olive extract and it was very low with higher concentrations, 1.5 and 2% v/v (Table 3.11).

Death rate of *Salmonella enteritidis* in home made mayonnaise

Fig. 3.43. The effect of ethyla acetate extract of green olives on the growth of *Staphylococcus aureus* in milk at 37°C determined by a Malthus Instrument



Each point, average of four observations

Table 3.11. The effect of the addition of ethyl acetate extract of green olives (0, 0.5, 1, 1.5 & 2% v/v) on *Staphylococcus aureus* S-6 in milk at 37°C after 48h of incubation (initial inoculum log_{10}7cfu/ml)

Extract (%v/v)	Detection time (h)	Viable counts log_{10}cfu/ml	Toxin production (ng/ml)
0	5.5	9.04	200
0.5	8.2	9.1	100
1	16.5	8.6	100
1.5	26.8	8.2	5
2	26.8	7.5	0.5

The death rates of salmonellas in mayonnaise made with virgin olive oil (Greek or Italian) were faster than in those containing blended olive or sunflower oil (Table 3.12). No viable *Salm.enteritidis* was recovered from mayonnaise made with virgin olive oil after 72h incubation but they were present in those made with blended or sunflower oil and incubated at 20° C for 3 d (Fig. 3.44). When all four experiments are considered (Table 3.12), the mayonnaise made from sunflower oil was always the least toxic. On two occasions the death rate of *Salm. enteritidis* in mayonnaise made from blended oil was not significantly different from that containing sunflower but on two occasions the former was faster than the latter. In all cases the death rates in mayonnaise made from extra virgin olive oil were significantly faster than those containing sunflower or blended oils.

Analysis of oils

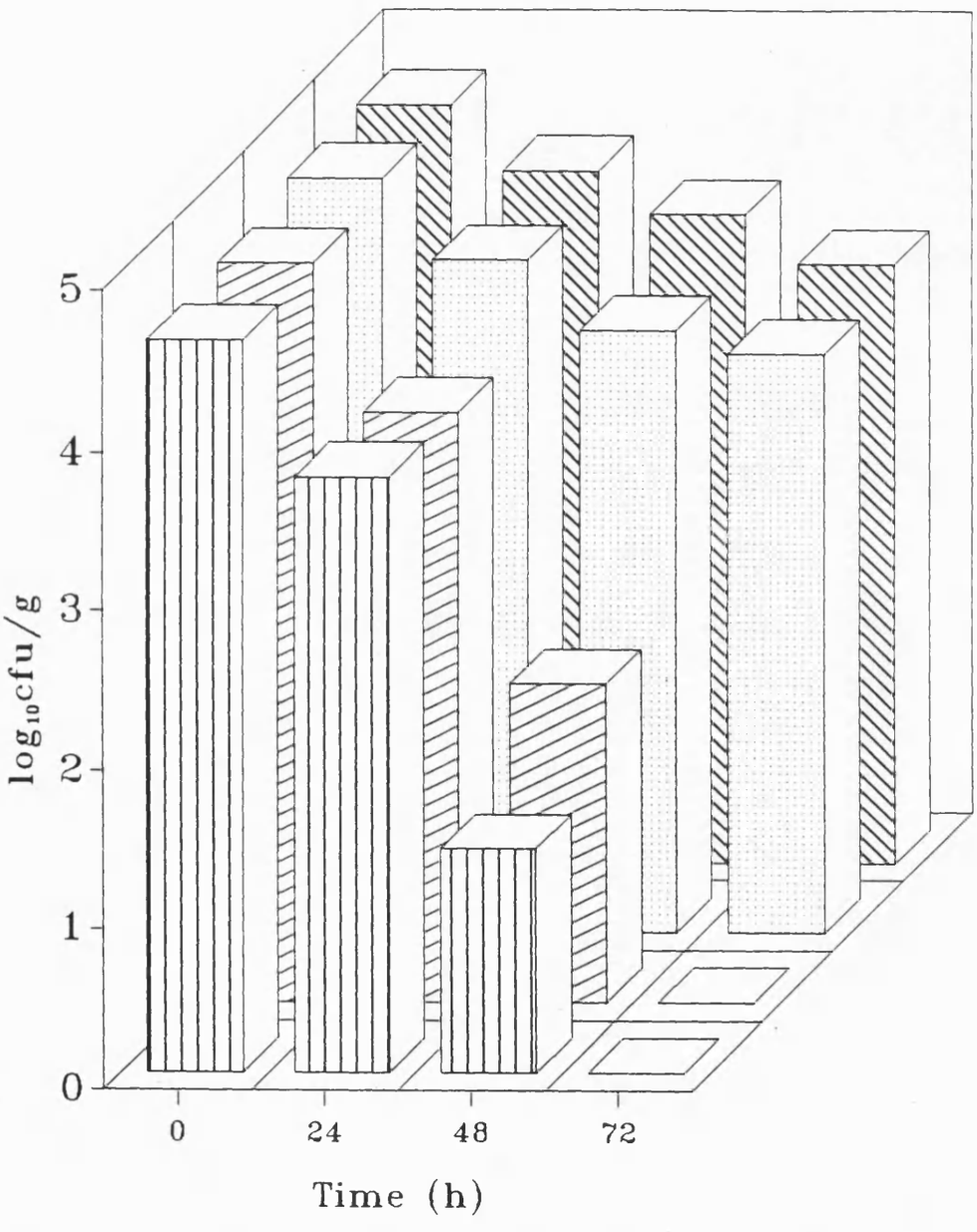
There were significant differences ($P<0.1\%$) in the acidity of oils used - *viz.* sunflower (0.2%; least acid), blended olive oil (0.4%) and the two extra virgin olive oils (0.5%; most acid) - as well as their range of phenolic compounds. The blended olive oil and sunflower oil contained insignificant amounts of such compounds whereas the extra virgin olive oils contained the greatest range (Chapter 2, Fig 2.11). Tyrosol occurred at different concentrations (peak 6; Fig 2.11) and oleuropein (peak 13) only in the virgin olive oils, its concentration being greatest in that of Greek origin. This oil contained phenolic substances identified with rutin (peak 10) and vanillic acid (peak 7). Traces of protocatechuic acid (peak 3) occured only in the Italian virgin olive oil. Two unidentified peaks (4 and 16) were present in the virgin olive oils of both origins. It is very probable that peak 4 is hydroxytyrosol, a derivative of oleuropein.

Table 3.12. The effect of different vegetable oils on the death rate of *Salmonella enteritidis* PT4 in mayonnaise incubated at 20°C

	Experiment			
Oil used	1st	2nd	3rd	4th
F-test*	***	***	***	***
Sunflower oil	x	x	x	x
Blend of olive oils	x	y	x	y
Italian olive oil (virgin)	y	nd	y	nd
Greek olive oil (virgin)	y	z	y	z

***: significant at 0.1% probability level
& : means with the same letter did not differ significantly
nd: not determined

Fig. 3.44. Fate of *Salmonella enteritidis* in home made mayonnaise with different oils incubated at 20°C



DISCUSSION

The use of different solvents for the extraction of phenolics could affect the total phenolic contents in the extracts and hence the extent of the antimicrobial activity (Rodriguez *et al.* 1988; Perez *et al.* 1992). Although the major total phenolic content was present in ethyl acetate extracts, the most peaks and most effective antimicrobial extract were obtained with propanolic ones (Rodriguez *et al.* 1988; Perez *et al.* 1992). Rodriguez and his collaborators (1988) concluded that at present the antibacterial effect cannot be correlated with a particular component of olive extract. Indeed correlation of inhibition with oleuropein is difficult because of the doubtful purity of the material used in previous studies (Garrido- Fernandez & Vaughn 1978; Gourama *et al.* 1998). This was confirmed in my study as well. My results with HPLC (see Chapter 2) showed that the commercial substance from a manufacturer in France was not pure. It was also confirmed by the manufacturer that their material is only 80% pure.

The suggestion that oleuropein has antimicrobial activity has been made by several workers (Fleming & Etchells 1967; Juven *et al.* 1968a; Ruiz-Barba *et al.* 1991). Others, however, have been unable to demonstrate such activity (Fleming *et al.* 1973). Indeed, Garrido-Fernandez and Vaughn (1978) reported that oleuropein at concentrations of 0.2% and 0.4% (w/v) could be utilised in a basal medium by microorganisms associated with olive fermentations but only after a lag phase of 2-5 days.

In the present study low concentrations (0.1-0.2% w/v) of oleuropein delayed by up to 30h the onset of growth of *Staph.aureus* in NZA or BHI whilst higher concentrations (0.4-0.6% w/v) were bactericidal. These results were obtained with a Malthus instrument which measures changes in electrical conductance in microbiological media resulting from bacterial growth and accumulation of metabolites (Firstenberg-Eden & Eden 1984). During growth, uncharged or weakly charged substrates (proteins and

carbohydrates) are converted into highly charged end products (amino acids, lactate etc.) resulting in increased conductance of the growth medium. In the present study the growth of *Staph.aureus* S-6 caused an increase in conductance of both NZA and BHI. The increase in conductance in NZA over the course of the growth cycle was almost twice that of the same cultures in BHI. In a comparison of the two media, Nychas *et al.* (1991) showed that, while BHI contained higher amounts of proteins, NZA - a pancreatic digest of casein - contained higher concentrations of readily available nitrogen sources including amino acids, small peptides and ammonia. The availability of the latter compounds to bacteria probably contributes to the greater change in conductance observed with cultures grown in NZA. In both media the pattern of growth was similar, i.e a rapid initial increase in conductance in the first 7-8h followed by a much slower one over the next 1-2d. The initial growth phase in NZA however lasted approximately 5h and resulted in an increase in conductance of 400ms compared to a growth phase of 2h and a change in conductance of 200ms in BHI. Despite these differences the final numbers of viable organisms in both media were similar.

In the case of *Salm.enteritidis* oleuropein had a bacteriostatic rather than a bactericidal effect. Indeed in all cases with *Salm. enteritidis* a medium with or without oleuropein resulted in almost equal final "growth" as expressed in microsiemens (conductance). A similar effect of another phenolic compound, BHA, on *Salm.typhimurium* has been noted by Pierson *et al.* (1980). They noted, however, an apparent decrease and subsequent increase in the number of viable cells of this microorganism, which they explained as an injury-recovery phenomenon. With *Salm. enteritidis* the addition of sodium chloride to Coliform broth did not act synergistically with oleuropein. In contrast the inhibition of *Staph. aureus* by BHA was influenced (synergistically) by sodium chloride (Robach & Stateler 1980).

In both the cases of growth inhibition mentioned above (*St. aureus & S. enteritidis*), pH affected growth inhibition by phenolic compounds (Tables 3.8 & 3.10). In similar studies with phenolic antioxidants such as BHA, it was found that growth inhibition at pH 3.5 was greater than that at pH 5.5 with *Aspergillus flavus*. Similarly the growth of *Clostridium perfringens* was more adversely affected at limiting pH values of growth, 5.5 and 8.5 (Wanda *et al.* 1976). The growth of *Staphylococcus aureus* was inhibited most at pH 8-9 (Ayaz *et al.* 1980). Similar results were obtained in my study with both bacteria tested (Figs 3.16-3.21 & 3.34-3.39).

In addition to the bactericidal or bacteriostatic effects noted above, Rodriguez *et al.* (1988) claimed that their propanol extract inhibited sporulation and germination of *B.megaterium*. In the former case they noted about a half-log decrease during an 8-h incubation of a mineral medium seeded with spores collected from a 3-day-old culture. When propanolic extract was present, there was no change in spore counts. The counts were done with a Petroff-Hausser chamber but apparently no effort was made to distinguish between phase bright and phase dark spores. Inhibition of sporulation was judged by doing viable counts on pasteurized (80°C for 10min) 3-day-old culture in medium with or without propanolic extract. In the first instance 90% of the counts were attributed to spores whereas in the second the proportion was reduced to 45%. Whether or not the latter were remnants from the inoculum is not clear. Our studies were focused on the action of extract of olive fruit as well as of oleuropein on the phase change and outgrowth of spores of *B.cereus* T. It was evident that at effective concentrations the supplements affected both spore germination and subsequent outgrowth (Figs 3.20, 3.22 & 3.23). The inhibition, therefore, occurs at the very primary stage (transformation of the phase bright spore to a phase dark form) of the development process from spore to vegetative cell. Moreover, it was found that the addition of oleuropein or olive extract at various times during the process of germination inhibited the outgrowth of

the germinated spores (Figs. 3.22 & 3.24).

Fats, proteins (Rico- Munoz & Davidson 1983), salts and temperature (Stern *et al.* 1979; Wanda *et al.* 1976) are among other factors which affect the preservative action of antioxidants. In this study the antimicrobial compounds extracted from olives and their effect on a model food system was examined with sterile reconstituted milk. The addition of phenolics inhibited the growth of and toxin production by *Staph. aureus*. Similar results were also found with ground turkey with 0.1% sorbate plus 100ppm BHA incorporated in the product. Little inhibition of the growth of *Staph. aureus* occurred in Genoa sausage fermented at 27°C in the presence of 0.003% BHA and BHT (Raccach and Henningsen 1981; cited by Kabara 1991). Although Rico Munoz and Davidson (1983) reported that the antimicrobial activity of a phenolic compound (BHA) was reduced dramatically in the presence of casein, Payne *et al.* (1989) found that a variety of phenolic compounds were very effective in inhibiting *Listeria monocytogenes* in a model milk system. Indeed Rico-Munoz and Davidson (1983) examined the effect of corn oil on the antimicrobial activity of BHA and TBHQ (2-tertiary butylhydroquinone). Whereas small amounts (1.5-3.0 %) of corn oil greatly reduced the effectiveness of BHA, larger amounts (4.5%) had a smaller effect on TBHQ.

In fat-rich food systems eg. mayonnaise, there is a greatly expanded oil/water interface which exerts a marked influence on the distribution of long-chain free fatty acids and phenolics between the aqueous and oil phases (Cornell 1979; Collins 1985). It is well known, also, that long- and/or short-chain fatty acids as well as phenolic compounds act as antimicrobial agents on food-borne bacteria (Freese *et al.* 1972; Branen *et al.* 1980). These substances could well affect the fate of foodborne pathogenic bacteria in mayonnaise. Edible oils contain both phenolic and long-chain fatty acids in various concentrations.

The concentration of long-chain fatty acids was the main difference between olive and sunflower oil. Although oleic (18:1) and linoleic (18:2) acids are present in both, the former is dominant in olive and the latter in sunflower oil (Kiritsakis 1988). In the present study the acidity (free fatty acids) in the two virgin olive oils was higher than that in the sunflower oil. The type and the concentration of phenolic compounds found in the oils differed significantly (Fig. 3.37). These differences presumably reflect the extraction methods, the variety of olives used for extractions as well as the period and the storage history of the oil (Kiritsakis 1988). In general olive oil extracted by mechanical means contains phenolic compounds in the range of 50-157 ppm whilst solvent-extracted oils contain 321-574 ppm (Kiritsakis 1988). The presence of the phenolic compounds in Greek or Italian virgin olive oil in this study has been reported by other workers also (Solinas *et al.* 1975; Graciani-Constante & Vazquez-Roncero 1980; Tsimidou *et al.* 1992a,b). It needs to be noted, however, that emphasis has usually been given to the antioxidant activity of the phenolic compounds in olive oils or to their contribution to organoleptic properties rather than their antimicrobial activity.

Although the antimicrobial activity of phenolic compounds in waste waters from olive oil mills ("katsigaros" in Greek, "alpechines" in Spanish) is well established, the mechanism of action by which these phenolic compounds inhibit the growth of microorganisms has not been elucidated. Judging from the studies of changes in soil bacteria, spore-forming bacteria (eg *Bacillus*) may be more sensitive than non spore-forming (Moreno *et al.* 1987; Paredes *et al.* 1986, 1987). In general it is well known that during endospore outgrowth the principal changes occurring are the initiation of RNA, protein and membrane syntheses (Vinter 1970). It has been found that the addition of phenols to bacteria, whether sporulated or not, perturbs the cytoplasmic membrane of the microorganisms (Fogg & Lodge 1945;

Davidson & Branen 1980; Blank *et al.* 1987; Rico-Munoz *et al.* 1987). Degre and Sylvestre (1983) suggested that BHA is a phenol and this class of compounds is known to act at the membrane level. Indeed it was found that phenols such as BHA, BHT, *p*-coumaric and caffeic acid attack the cytoplasmic membrane destroying its permeability and causing leakage of intracellular constituents such as glutamate, potassium and phosphorus or inhibiting such membrane functions such as electron transport and nutrient uptake, ATPase activity etc (Judis 1963; Juven *et al.* 1972; Eklund 1980; Degre *et al.* 1983a,b; Rico-Munoz *et al.* 1987; Ruiz-Barba *et al.* 1990; Denyer & Hugo 1991; Hugo 1991). In particular Degre and Sylvestre (1983) found that the mode of action of BHA against *Staph. aureus* was related to the leakage of nucleotides. According to Hugo (1991) leakage is known to be a general phenomenon induced by many antibacterial substances. BHA being a highly hydrophobic molecule, the cytoplasmic membrane is likely to be the main site of adsorption since the cell wall of *Staph. aureus* contains as little as 1 or 2% lipid material. The low water solubility of many phenolics (eg BHA, oleuropein etc) probably precludes any significant diffusion into the cytoplasm. Moreover low temperature decreases the solubility and hence the concentration of the phenol in the cell membrane lipid (Wanda *et al.* 1976). While this is one possibility, the effect of temperature on rates of reaction is probably more important. It has been reported that no inactivation of cells occurred at 0°C with 0.02m BHT, whereas a 1000-fold reduction in bacterial numbers was observed at 25°C (Wanda et al. 1976). Lysis of bacterial protoplasts by BHA occurred at concentrations lower than those causing leakage of nucleotides from whole cells (Degre and Sylvestre 1983). This is an indication that the cell wall might play an important role in the relative resistance of whole cells lysis by low concentration of phenolics. Similar conclusions have been drawn by Prindle and Wright (1977) who noted that the mode of action of phenols is concentration dependent. Low concentrations affect the activity of enzymes associated with energy

production whilst high amounts caused a precipitation of proteins. In contrast Judis (1963) speculated as to whether or not the alleged damage caused to the cell membrane is quantitatively related to the amount of phenol derivative to which the cell is exposed or whether the effect is such that once small damages are caused, the lesions enlarge and leakage proceeds continuously.

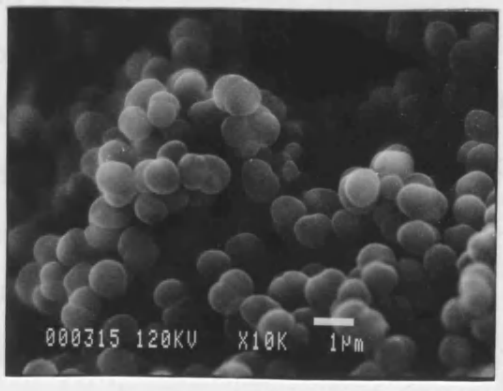
Since in our study the different protein patterns, as shown by either HPLC or SDS-page electrophoresis, could not be attributed to protein degradation by the medium - because of the lack of detectable high molecular weight protein - these differences could be due either to significant changes in the exoprotein excretion of *Staph. aureus* in the presence of the various concentrations of oleuropein or to protein (eg. cytoplasmic) precipitation by oleuropein. Indeed it has been shown that phenolic antioxidants are able to bind proteins, most probably through hydrophobic interactions (Rico-Munoz *et al.* 1987).

The HPLC and SDS-page electrophoresis results in this study suggest that phenolic compounds from olives are membrane-active agents possibly because they affect permeability. However, extracellular material, as demonstrated with HPLC, is only a measure of a generalized loss of membrane function and it is more likely that these compounds interfere with energy metabolism, synthesis of macromolecules or actively growing cell membranes. With the last mentioned view, the use of scanning electron microscopy in the study of Ruiz-Barba *et al.* (1990) showed that the whole cells of phenolic-untreated *Lact. plantarum* were smooth compared to the cells treated with phenolics for 24 h. In the latter case the bacterial surfaces become irregular and rough. In my study similar results were obtained with *B.cereus* and *Staph.aureus* (Fig 3.45a,b).

The bactericidal/bacteriostatic effect of phenolic compounds is shown by perturbations at two different levels: cell wall integrity and the physiology of bacteria. Indeed as far as it concerns the second level of physiological

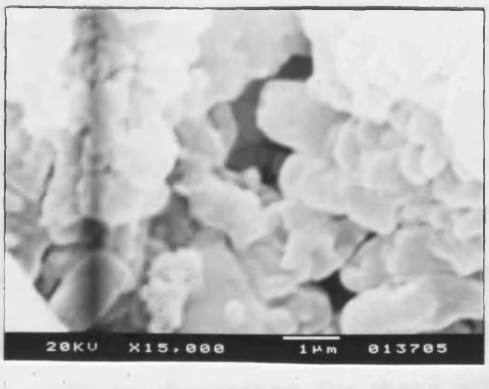
Fig. 3.45. Cells of *Bacillus cereus* T and *Staphylococcus aureus* S-6 without (a & b respectively) or with (c & d) addition of commercial oleuropein and incubation at 30°C for 24h, as shown by electron microscopy

a



b

c



d

alteration, it was shown in this part of my study that they affected *Staphylococcus* enterotoxin B (SEB) and lactate production as well as the rate of glucose assimilation. Moreover it has been reported that the production of SEB is unaffected over the pH range 6.0-6.9 (Genigeorgis & Sadler 1966). In my samples, the pH was not changed by the addition of oleuropein or the production of lactate. Nychas *et al.* (1991) showed that the addition of glucose to both NZA and BHI resulted in a marked decrease in pH, due to L-lactate production, which may be responsible for the repression of SEB synthesis (Metzger *et al.* 1973). If pH was the key factor, inhibition would be expected only in samples lacking oleuropein, where there was a marked production of lactic acid (Table 3.2) compared to samples with oleuropein. In this study the formation of L-lactate and enterotoxin as well as glucose assimilation were determined by the amount of oleuropein added (Table 3.2). This was pronounced with low concentrations of oleuropein where there was no significant differences in viable counts among samples with oleuropein (0.1% oleuropein, 6.5 x 10^8cfu/ml; 0.2% oleuropein, 5 x 10^8cfu/ml) or without (control, 9.1 x 10^8cfu/ml). Inhibition of enterotoxin A production by *Staph.aureus* strain 100 together with inhibition of growth by BHA was also noted by Ayaz *et al.* 1980.

The decrease in the percentage of glucose utilisation as well as the reduction in the formation of L-lactate could be due to the inhibitory effect of oleuropein on substrate uptake or on the electron transport chain. This effect was more pronounced in NZA than in BHI. The different types and amounts of proteins in these two media could be the reason for these effects, as it is well known that proteins mask the inhibitory effect of phenolics (Ruiz Barba *et al.* 1991). The activity of phenolics from olives could be decisive also for the spores, since the effectiveness of germination inhibitors may depend upon their ability to permeate the spore coat and block germination-promoting sites (Cook & Pierson 1983). Moreover, phenolic compounds, such as chlorocresol which have been tested against spore

germination, may cause denaturation of germination enzymes, inhibit the lytic enzyme subtilopeptidase A (Parker & Bradley 1968), or interfere with the use of L-alanine or other amino acids necessary for the initiation of the germination process (Sierra 1970).

As the food industry searches for naturally occurring antimicrobial compounds further investigations into the effect of phenolic compounds such as oleuropein should be carried out. The effect of oleuropein in combination with other antibacterial agents such as NaCl should be examined, in more detail, particularly since its effect appears to be accentuated in the presence of 5% (w/v) NaCl (Fleming *et al.* 1973). Furthermore it has been reported recently that oleuropein retains activity even after heating at 121°C/15 min (Ruiz-Barba *et al.* 1991) which would suggest its potential use in heat processed foods.

CHAPTER 4

MICROBIOLOGY OF OLIVES

Introduction

As far as can be ascertained there is limited information about the microbiology of olives on trees in the groves or during storage prior to oil extraction. A survey of the microbial population on the surface of olive fruits before harvesting was done in this study and various yeasts, pseudomonads and lactic acid bacteria were isolated. A similar study was done by Pelagatti (1978-1980) who immersed olives of 12 cultivars in selective media. The microorganisms he isolated are listed in Tables 4.1 & 4.2. Ercolani (1978) isolated many species of aerobic, heterotrophic, mesophilic bacteria from olive leaves (Table 4.3). He noted that there were seasonal fluctuations in the composition of the phylloplane of olives and showed that *Pseudomonas savastanoi* was the dominant organism on the leaf surfaces of olive trees (Ercolani 1979). He subsequently studied in detail this causative organism of the disease, olive knot or tubercle, which occurs in most regions of the world where *Olea europaea* is grown (Ercolani 1983, 1985). The disease gets its name from the woody outgrowths that are found most frequently on young stems and on branches and twigs as a consequence of wound infections. Extensive studies have shown that knot development involves: colonization of the infection site, formation of lysogenous cavities and finally abnormal enlargement and proliferation of the host cells. The developmental pattern of hypertrophy and hyperplasia suggests that they are induced by a substance with cytokinin-like activity synthesized by *Pseudomonas savastanoi* (Surico *et al.* 1976). The disease is associated with a reduction in yield and quality of

Table 4.1. Lactic bacteria isolated from green olive fruits

Lactobacillus
delbrueckii
leichmannii
helveticus
acidophilus
casei subsp. *alactosus*
casei subsp. *rhamnosus*
xylosus
plantarum
curvatus
coryniformis subsp. *coryniformis*
fermentum
brevis
hilgardii

Based on Pelagatti (1978-80)

Table 4.2. Yeasts isolated from green olive fruits

Brettanomyces spp.	*Rhodotorula*
anomalus	*aurantiaca*
Candida	*glutinis* var. *glutinis*
guilliermondi	*mucilaginosa*
olea	*rubra*
parapsilopsis var. *intermedia*	*Saccharomyces*
tenuis	*bayanus*
utilis	*cerevisiae* var. *ellipsoideus*
Cryptococcus	*chevalieri*
albidus var. *albidus*	*exiguus*
laurentii var. *laurenti*	*fermentati*
macerans	*globosus*
Debaryomyces	*italicus*
hanseii	*kluyveri*
nicotianae	*oleaceus*
Endomycopsis vini	*rosei*
Hanseniaspora	*rouxii*
osmophila	*uvarum*
uvarum	*Schizosaccharomyces* spp.
valbyensis	*octosporus*
Hansenula	*Sporobolomyces roseus*
anomala var. *anomala*	*Torulopsis*
anomala var. *schneggii*	*candida*
holstii	*glabrata*
Kloeckera	*holmi*
apiculata	*magnoliae*
corticis	*stellata*
Kluyveromyces veronae	*Trichosporon pullulans*
Leucosporidium spp.	
Metschnikowa pulcherrima	
Pichia	
fermentans	
membranefaciens	
pinus	
polymorpha	
terricola	
vini var. *vini*	

Based on Pelagatti (1978-1980).

Table 4.3. Bacteria isolated from olive leaves during surveys done in two different years

Bacteria	Distribution (%)	
	1979	1991
Pseudomonas		
savastanoi	67.86	
subsp. *syringae*		51.00
delafieldi	0.11	
fluorescens	1.06	3.90
aeruginosa	-	0.04
Bacillus spp.	-	0.29
subtilis	0.34	0.57
megaterium	4.02	3.80
Serratia marcescens	1.34	0.81
Erwinia spp.	-	0.37
carotovora	-	0.08
Pantoea		
agglomerans	8.50	6.00
Klebsiella		
pneumoniae	1.40	-
planticola	-	1.20
Lactobacillus plantarum	1.45	2.80
Leuconostoc		
dextranicum	1.12	-
mesenteroides	-	
subsp.*dextarnicum*		3.10
Acetobacter aceti	1.23	4.70
Arthrobacter globiformis	2.07	1.40
"Micrococcus luteus"	3.63	2.20
Xanthomonas compestris	3.35	6.70
Gluconobacter oxydans		4.30
Curtobacterium plantarum		2.20
Enterococcus faecium		1.20
Clavibacter sp.		0.98
Micrococcus sp.		0.82
Cellulomonas flavigena		0.40
Zymomonas mobilis		0.30
Alcaligenes faecalis		0.27

Based on Ercolani (1978, 1991)

olives. *Pseudomonas savastanoi* causes a similar disease on other plants, including *Fraxinus excelsior* (ash), *Nerium oleander & Ligustrum japonicum.*

In studies made between 1984 to 1989, Ercolani (1991) confirmed and extended the inventory of mesophilic, heterotrophic, aerobic or facultatively anaerobic bacteria in the phylloplane of the olive which he made between 1973 to 1975. The identity of isolates in the latter survey is listed in Table 4.3. He concluded that the composition of bacterial communities on the phylloplane in general had not undergone significant long term changes during the period 1973 to 1989. He assumed that technical rather than biological reasons accounted for the appearance in the second study of several new taxa, i.e. *Alcaligenes faecalis, Bacillus* spp., *Cellulomonas flavigena, Clavibacter* spp., *Curtobacterium plantarum, Erwinia carotovora, Erwinia* spp., *Gluconobacter oxydans, Klebsiella planticola, Micrococcus* spp., *Pseudomonas aeruginosa, Streptococcus faecium* and *Zymomonas mobilis.*

There is considerable information about the microflora, especially its lipolytic activity, of waste products from olive oil extraction mills. The lipolytic activity, has been studied in detail because of its detrimental effects on olive oil. As yet, however, no clear-cut conclusion can be drawn. Oil hydrolysis mediated by lipases and the resultant oxidation (rancidity) affects the organoleptic properties of the final product. Venezia and Sallusto (1933) reported that olive fruits contain a lipase and Cantarelli (1960) noted that the flesh contains both a lipase and lipoxidase, the latter occurring in the endocarp. According to the latter author, the increase in the titratable acidity of olive oil during storage is attributable to enzymic action rather than autocatalysis. Borbolla y Alcala *et al.* (1958) could not verify the existence of a lipase or a lipoxidase in healthy olives. Consequently they doubted the existence of such endogenous enzymes and concluded that the lipolytic

enzymes noted by Florenzano (1951) were probably derived from microorganisms which grew in the olive paste at various stages of processing or even in the oil produced therefrom. Suarez Martinez (1975) reported that enzymatic lipolysis could be caused by the endogenous enzymes in the fruit, pulp and seed. It is also well known that the microorganisms which grow on the stored olives before processing, in the olive paste before extraction with solvents, or on the olives attacked by the insect, *Dacus oleae*, are commonly lipolytic as shown by studies done in Spain and Italy (Balatsouras 1966). Thus Verona and Valleggi (1949) isolated many lipolytic microorganisms, mainly yeasts but bacteria and moulds as well, from heaps of pits (endocarps) from olive oil mills. Lipolytic activity of microbial origin increased the acidity of oil produced from pits *vis* α *vis* the virgin material. The yeasts isolated by these workers are listed in Table 4.4. In 1954 Verona isolated a new genus, *Trichosporon,* from pit samples at olive oil factories. It produced lipase and split olive oil into glycerine and free fatty acids. Florenzano (1952) also studied the microflora at various stages of oil processing. He isolated moulds, bacteria and yeasts. These were mainly lipolytic. Later Montefredine (1957) demonstrated that the majority of the moulds isolated from heaps of pits produced lipase.

Gonzalez-Cancho (1957a) isolated moulds, yeasts and bacteria (Table 4.4) from olives stored in heaps in readiness for oil production. He noted that *Serratia* and *Pseudomonas* spp. were the most lipolytic genera followed by members of the genera *Trichosporon* and *Pichia*. All of the other microorganisms were lipase negative. In another study Gonzalez-Cancho (1957b) found geographical differences in the composition of the microflora and he isolated additional species of bacteria, yeasts and moulds (Table 4.4). The lipolytic strains are highlighted in this Table. The Italian Picci (1959) isolated bacteria, yeasts and moulds from the oviposition site of *Dacus oleae* on olives (Table 4.4). He concluded that the majority of the isolates

Table 4.4. The microbiology of olives. Stored olives (a); oviposition sites of *Dacus oleae* (b); and heaps of pits (c).

a	*b*	*c*
Moulds		
Rhizopus	*Rhizopus nigricans**	*nd*
Aspergillus	*Actinomucor**	
*flavus**	*Oospora**	
glaucus	*Aspergillus glaucus**	
*niger**	*Fusidium**	
*sydowi**	*Geotrichum candidum**	
*terreus**	*Penicillium*	
*versicolor**	*purpurescens**	
Fusarium	*Alternaria tenuis**	
Penicillium	*cheiranthi**	
*notatum**	*Pullalaria pullulans**	
purpurogenum	*Cladosporium*	
Alternaria	*avellaneum**	
	*Fusarium vasinfectum**	
Yeasts		
Pichia	*Saccharomyces*	*Rhodotorula*
fermentans	*cerevisiae**	*mucilaginosa**
membranefaciens	*Pichia fermentans*	*Candida*
Saccharomyces	*Debaryomyces*	*lipolytica**
italicus	*kloeckeri**	*guillermondii**
elegans	*sybglobosus**	*parapsilopsis*
Candida	*Candida guillermondi*	*var.intermedia**
*crusei**	var.*membranefaciens**	*Trichosporon*
parapsilopsis	*intermedia**	
*var.intermedia**	*parapsilopsis**	
*Trichosporon sericum**	*parapsilopsis*	
	*var.intermedia**	
	*pulcherrima**	
	*tenuis**	
	*Rhodotorula glutinis**	
	*mucilaginosa**	
	Trichosporon	
	behrendii	
	Cryptococcus	
	laurentii	
Bacteria		
Aerobacter	*Micrococcus*	
Escherichia	*roseus**	nd
*Serratia marcencens**	*flavus**	
*plymuthicum**	*Sarcina lutea**	
Pseudomonas	*Bacillus*	
*aeruginosa**	*cereus**	
Achromobacter	*brevis**	
Bacillus subtilis	*megaterium**	
megaterium	*pumilus**	
cereus	*subtilis**	

*: Lipolytic strains
nd: not detected
Based on Verona & Vallegi (1949), Gonzalez-Cancho (1957; 1957a) and Picci (1959)

produced lipase. The exceptions were *Pichia fermentans*, a strain of *Cryptococcus laurentii*, *Trichosporon behrendii*, some species of *Penicillium* and *Alternaria*. Gracian *et al.* (1961) analysed samples chemically from olive pits, the same heap being sampled at various times before processing. They determined the quantity and quality of the oil by physical and chemical methods. They found that by the time hydrolysis of fatty substances had occurred, a decrease in the weight of oil had occurred also. Both phenomena were attributed to microorganisms growing within and on the surface of the heap, and splitting the glycerides to glycerine and free fatty acids. The microorganisms used the hydrolysis products for growth thereby causing not only deterioration of the quality but also loss in the yield of oil. They found also an increase in the acidity of the oil and the formation of hydroxyacids as well as other metabolites which were transfered eventually to the oil of the endocarps. The lipolytic enzymes produced by many microorganisms on the raw product are found in the final product even after UHT sterilization. Consequently they can cause spoilage of the sterile product.

To act optimally the lipase needs an olive oil of pH 8-8.5 and a temperature of 40°C (Adams 1981a,b). There is a high probability also of these enzymes surviving processing during the macerating of olives (done at 35-40°C) before oil extraction. Thus they can be transferred to the olive oil where they exacerbate the development of oxidative rancidity.

Lipases from *Candida rugosa* (Han 1985), *Saccharomycopsis lipolytica* and *Micrococcus caseolyticus* (Jonsson 1976) have been shown to hydrolyse olive oil to a great extent. The hydrolysis by the lipase of *Sacch.lipolytica* was highly specific for oleic acid while that of *M.caseolyticus* was totally nonspecific (Jonsson 1976). Small amounts of oleic acid activate but higher concentrations inhibit the lipase of *Candida lipolytica* (Ota *et al.* 1972). Olive oil stimulates the growth of *Achromobacter lipolytica* thereby increasing lipase production and hydrolysis (Kahn *et al.* 1967). The same is

true for *Pseudomonas mephitica* (Kosugi *et al.* 1971). Olive oil stimulates lipase production initially by enhancing growth of *Geotrichum candidum* but eventually the release of glycerol from the triglycerides diminishes lipase production (Nelson 1953; Wouters 1967). Lipases of *Pseudomonas fragi, Staph. aureus, Aspergillus niger* also hydrolyse olive oil (Nashif & Nelson 1953, Alford *et al.* 1961, Fukumoto *et al.* 1963).

MATERIALS & METHODS

Sampling

In order to isolate and study the microorganisms on the surface of olive fruits and vegetation, samples were taken during two harvesting periods. The samples were olives and leaves from the tree as well as olives from the ground, in different regions of Greece, Crete (Heraklio, Chania, Rethimno), Peloponnesos (Sparti, Kalamata), Naxos and Spata (a place near Athens). The olives were classified according to variety (Koroneiki, Tsounati, Lianolia, Kalamon, Throumpolia), the stage of maturity (green or brown) and the degree of damage or infection (healthy, damaged or infected) (Table 4.5).

Microbiological analysis

In the field

The cotton stick swab technique was used in this part of the study. The swab was immersed aseptically in sterile 1/4 Ringer's solution and used to transfer the endogenous flora onto selective media. This technique was used for qualitative but not for quantitative determination of the endogenous microflora.

In the laboratory

Samples were collected aseptically in plastic bags and stored under refrigerated conditions during transfer to the laboratory. Olives (25g) were immersed in 225ml of Ringer's solution and shaken for 30min. After the preparation of decimal dilutions, quantities (1ml or 0.1ml) -depending on the

Table 4.5. Area, variety and sampling

Year	Place of sampling	Variety	Samples taken from	Maturity of olives	Physiological stage
1987	Chania Heraklio Naxos	Koroneiki Throumpolia Tsounati Lianolia	leaves olives soil	green brown or black	healthy damaged
1988	Chania Rethimno Kalamata Sparti Spata (Athens)	Koroneiki Throumpolia Tsounati Kalamon	leaves olives soil	green brown or black	healthy damaged

technique- were inoculated on Petri dishes containing the media listed in Table 4.6.

Identification

a. Bacterial identification

In general five representative colonies were randomly picked from the appropriate media (Pseudomonas CFC Agar, MRS Agar), purified by streaking on the same medium and finally maintained until required at 4°C on the same medium. The organisms were tested for the Gram reaction and bacterial characterization and identification were done with API tests. Bacterial isolates were identified by using the methodology described by API NE and 50 CH system for pseudomonads and lactic acid bacteria respectively.

b. Yeast identification

Ten representative yeast colonies were randomly selected from plates of Chloramphenicol Rose Bengal agar. The yeasts were purified by streaking on the same medium and finally maintained until required at 4°C on the same medium.

The following tests were used for the identification of yeasts: anaerobic fermentation of glucose, maltose, sucrose, a-D-trehalose, melibiose, lactose, D-cellobiose, melezitose, raffinose (Harrigan & McCance 1976), resistance to different concentrations of actidione (0.1 and 0.01%) (Harrigan & McCance 1976), and growth at different temperatures (4, 25 and 32°C).

Table 4.6. Media, incubation period, temperatures and techniques used in this study

Group or organism	Medium*	Incubation	
		Period (d)	Temperature (°C)
Total Viable Count	PCA (s)#	3	25
Pseudomonas spp.			
(Mead & Adams, 1977)	CFC (s)	3	25
Enterobacteriaceae	VRBG (p)	1	30
Yeasts	RBC (s)	5	25
Lactic acid bacteria	MRS (p)	5	25

* : from Oxoid when available, otherwise made from basic ingredients in the laboratory

: an inoculum was either spread on the surface of dried medium (s) or used to prepare pour plates (p)

Test for lipolytic activity

To test the lipolytic activity of microorganisms, the tributyrin test (Oxoid) was used as well as the Victoria blue agar medium. The latter was prepared as follows: yeast extract 3g/l, peptone 5g/l, agar 15g/l and olive oil 50 ml/l are mixed and the pH adjusted to 7.8. The medium was dispensed in 100ml amounts and sterilised at 121°C/15min. One ml of Victoria blue solution (6g/l) sterilised by filtration - was added to every portion and poured into Petri dishes. The test microorganisms were spot inoculated on the surface of the medium and, after 5 days incubation at 30°C, a distinct blue zone appearing under or around the colony was recorded as a positive lipase reaction.

RESULTS

A microbiological survey was done in 1987 and 1988. The sampling was done either with cotton swabs in the field or with immersion of the olives in Ringer's solution in the laboratory. In 1987 the survey was done mainly on the islands of Crete and Naxos during November. Total Viable Counts, as well as the enumeration of Pseudomonads, Enterobacteriaceae, lactic acid bacteria and yeasts/fungi were done in this survey using the appropriate selective/ differential media (Table 4.6). Isolates from selected colonies were purified and characterised in sufficient detail to achieve either a tentative or substantive identification. This was done not only to monitor the performance of a medium, but to establish whether or not a characteristic microbial association occurred on olives. *Pseudomonas* spp. [Gram negative, oxidase positive (Kovacs 1956), catalase positive, oxidative-positive (Hugh & Leifson 1953), mobile rods] and yeasts/moulds grew on Plate Count agar, the least selective medium used in the first phase of the study. Lactic acid bacteria (Gram positive rods) were the only organisms noted in routine examination of MRS medium and yeasts were the only organisms isolated on Rose Bengal Chloramphenicol agar.

During the first year (November 1987) of this survey a total of 68 (35 in laboratory conditions and 33 in the orchards) samples were analysed in order to determine the range of the microorganisms occurring on olives. It was found that bacteria growing on Mann-Rogosa-Sharpe medium, on Pseudomonas Agar supplemented with CFC and on Rose Bengal Chlorampenicol (yeasts & moulds) were common organisms on olives. Of the samples, 35 were analysed further. The results are shown in Table 4.7. It is evident that pseudomonads and yeasts were the dominant organims followed by the lactic acid bacteria. Indeed it was found that there was a statistically significant difference between the numbers of these organisms

Table 4.7. The mean value (log_{10} cfu/g) and the percentage contribution of microorganisms to the microflora on olives, the range (min/max) and the number of samples

1987

Microorganisms	Number of samples	Mean value	Range
Pseudomonads	31	6.08	4.0-8.50
Yeasts- Fungi	35	5.92	4.0-8.84
Lactic acid bacteria	26	5.03	3.6-7.35
Enterobacteriaceae	15	4.24	0.0-9.47

1988

Microorganisms	Number of samples	Mean value	Range
Pseudomonads	42	1.05	0.0-5.63
Yeasts- Fungi	42	4.95	2.7-6.41
Lactic acid bacteria	42	3.04	0.0-6.37
Enterobacteriaceae	42	0.68	0.0-4.32

(Table 4.7). In 9 out of 15 samples examined, Enterobacteriaceae were found to contribute in very significant numbers to the endogenous microbial flora (Table 4.7). In the remaining 6 samples, it was difficult to count even one colony in the lowest dilution on VRBG. It was evident that the place of sampling (Heraklion, Chania) affected significantly only the numbers of organisms growing on MRS medium (Table 4.8). The statistical analysis of the different olive varieties (one way analysis of variance, F-test, Table 4.9) however revealed that the contribution of a particular group of organism did not differ significantly between cultivars (Koroneiki, Tsounati, Throumpolia). There was a significant difference (Table 4.10) in the level of yeasts but not lactic acid bacteria among the different fruits (healthy, damaged, mature) of olives. Leaves and green healthy (undamaged) olives were found to have lower counts than damaged or undamaged black olives. This was also evident in the second year of the survey. The counts on leaves was higher than those on healthy or damaged green olives (Table 4.10).

A total of 42 samples were examined (Table 4.7) during the survey in the second year. This survey was done at different places and in different time (October) to that of the first year. In this case the microbial load on olives from Rethymno and Chania on the island of Crete, Peloponnesos (Sparti, Kalamata) and Spata (Athens) were studied. Some samples were found to have extremely low counts of pseudomonads, Enterobacteriaceae and lactic acid bacteria irrespective of the place of sampling. This phenomenon was not evident in the first survey (Table 4.7).

The counts of lactic acid bacteria in samples from Peloponnesos differed significantly ($P>0.5$) from those on samples from Athens and Crete (Table 4.8). The mean count of the yeasts during the first were higher than those obtained in the second survey (Tables 4.7 & 4.8). The most dramatic difference however concerned the level of pseudomonads and

Table 4.8. Differences between the populations of lactic acid bacteria, pseudomonads and yeasts/fungi on olives in different areas in two different years.

1987

Group of organisms	Place of sampling Heraclio	Chania	t-test
Lactic acid bacteria	4.47#x (11[1])	5.38y (12)	*
Pseudomonas spp.	6.02z (15)	6.27z (15)	ns
Yeasts/Fungi	6.02z (17)	5.81z (15)	ns
F-test	*	*	

1988

Group of organisms	Place of sampling Crete	Peloponnesos	Athens	F-test
Lactic acid bacteria	2.55x (10)	3.88z (8)	2.64x (12)	*
Yeasts/Fungi	4.81y (13)	5.19y (11)	4.87y (9)	ns
Pseudomonas spp.	- (11)	- (9)	- (10)	
F -test	**	**	**	

* : significant at the 95% level
** : significant at the 99% level
ns : non significant difference
\# : $\log_{10}$cfu/ml
1 : number of samples in parenthesis
x, y, z : numbers with the same letter x, y or z do not differ significantly
\- : no colonies were found on the selective medium used for pseudomonads

Table 4.9. Differences between the populations of lactic acid bacteria, pseudomonads and yeasts/fungi on olives, on different varieties of olives at two differnt years

1987

Group of organisms	Variety A	B	C	F-test
Lactic acid bacteria	5.10#x (9&)	4.94x (8)	4.80x (6)	ns
Pseudomonas spp.	6.10z (11)	6.45z (11)	5.46z (5)	ns
Yeasts/Fungi	5.88z (11)	6.01z (15)	6.03z (5)	ns
F -test	*	*	*	

1988

Group of organisms	Variety A	B	C	D	F-test
Lactic acid bacteria	3.5x (8)	3.6x (9)	3.3x (11)	2.7x (8)	ns
Yeasts/Fungi	5.24y(9)	5.40y(10)	4.58y(9)	5.00y(9)	ns
Pseudomonas spp.	- (10)	- (8)	- (10)	- (11)	
F -test	*	*	*	*	

A: Koroneiki B: Tsounati C: Throumpolia D: Kalamon
&: number of samples in parenthesis
#: mean values with the same indicator show not significant difference (LSD).
*: significant difference at 5% level
**: significant difference at 1% level
ns: non significant difference
- : no colonies were found on the selective medium used for pseudomonads

Table 4.10. The influence of the physiological condition of olives (damaged or healthy, green or black) and leaves on the microflora in two different years

1987

Olives	Microorganisms		
	Yeasts	Lactic acid bacteria	Pseudomonads
Black healthy	5.9# (5&)	4.92 (4)	6.10 (4)
Green healthy	4.92 (4)	4.62 (5)	5.46 (3)
Black damaged	6.57 (10)	5.51 (8)	6.54 (10)
Green damaged	6.51 (7)	4.92 (2)	6.41 (7)
Leaves	5.08 (6)	4.56 (5)	5.27 (6)
F-test	*	ns	ns

Year two (1988)

Olives	Microorganisms	
	Lactic acid bacteria	Yeasts
Green healthy	2.86 (22)	4.74 (19)
Geen damaged	3.26 (17)	4.39 (23)
Leaves	3.72 (15)	5.66 (15)
F-test	ns	**

*: significant difference at 5% level
**: significant difference at 1% level
ns: non significant difference
#: $\log_{10}$cfu/ml
&: number of samples in parenthesis

Enterobacteriaceae. In the second survey, the counts of both groups were extremely low. Statistical analysis of the different varieties revealed (Table 4.9) that there was no significant differences in the level of any organism among the varieties examined. Tables 4.11 and 4.12 summarize the main biochemical characteristics of lactic acid bacteria and yeasts respectively.

In the study of the isolates from *Pseudomonas* medium, an unidentified *Pseudomonas* spp., *Aeromonas sobria* and *Pseudomonas putida* were the dominant organisms (Table 4.13). The biochemical characteristics of these isolates as found with the API NE system are shown in Table 4.14. Only 22% of these bacteria produced proteases, while 86% of 75 isolates tested for lipolysis were found to be positive. The results obtained with pseudomonads on victoria blue agar media containing olive oil were essentially the same as those on tributyrin agar (Table 4.15). The number of strains of yeasts hydrolysing tributyrin were larger than those producing acid on victoria blue agar. Only 9.5 % of the lactic acid bacteria produced lipases (Table 4.15).

In this project I have provided some basic knowledge concerning the groups of organims existing on olives in orchards as well as on their physiological characteristics. Through the period of the survey I understood that the place and the way of sampling, the season, the maturity and the condition of olives could influence the microbial flora. It was important to note that the cultivation techniques (eg. irrigated or not orchards), the use of insecticides, the meteorological conditions in a specific area, the different cultivars, could also affect the groups of organisms. Similar results have been reported by Pelagatti (1978-80).

Table 4.11. Percentage of positive tests on API 50CH of 29 lactic acid bacteria isolated on MRS agar

Test substrate	% Positive	Test substrate	% Positive
1) Glycerol	-	26) Salicin	89.65
2) Erythritol	-	27) Cellobiose	72.41
3) D-Arabinose	-	28) Maltose	96.55
4) L-Arabinose	96.55	29) Lactose	89.65
5) Ribose	93.10	30) Melibiose	48.28
6) D-Xylose	41.38	31) Saccharose	100.00
7) L-Xylose	-	32) Trehalose	100.00
8) Adonitol	-	33) Inulin	-
9) β-Methyl-xylose	-	34) Melezitose	3.45
10) Galactose	93.10	35) D-Raffinose	10.35
11) D-Glucose	96.55	36) Amidon	-
12) D-Fructose	96.55	37) Glycogen	-
13) D-Mannose	100.00	38) Xylitol	-
14) L-Sorbose	-	39) β-Gentiobiose	17.24
15) Rhamnose	6.90	40) D-Turanose	20.69
16) Dulcitol	-	41) D-Lyxose	-
17) Inositol	-	42) D-Tagatose	3.45
18) Mannitol	68.97	43) D-Fucose	-
19) Sorbitol	-	44) L-Fucose	-
20) α-Methyl-D-mannose	-	45) D-Arabitol	-
21) α-Methyl-D-glucoside	41.37	46) L-Arabitol	-
22) N-Acetyglucosamine	96.55	47) Gluconate	34.48
23) Amyglaline	17.24	48) 2 keto-gluconate	-
24) Arbutine	86.21	49) 5 keto-gluconate	10.35
25) Aesculin	96.55		

Table 4.12. Test substrate and percentage of positive reactions of 50 yeasts isolated on Rose Bengal Chloramphenicol Agar

Test substrate	Kind of reaction	% Positive reactions
1. Glucose	fermentation (anaerobic)	100.0
2. D-lactic acid	#	96.0
3. D-maltose	#	96.5
4. Sucrose	#	100.0
5. α-D-trehalose	#	94.5
6. Melibiose	#	90.0
7. Lactose	#	85.5
8. D-cellobiose	#	98.0
9. Melesitose	#	93.5
10. Raffinose	#	94.0
11. Tributyrin	lipolysis	91.3
12. 32°C	growth	100.0
13. 25°C	@	100.0
14. 4°C	@	71.0
15. Actidione 0.1%	@	60.3
16. Actidione 0.01%	@	72.6

: fermentation
@ : growth

Table 4.13. Identification of 42 microorganisms isolated from CFC medium using the API 20NE* and the percentage contribution of every species

Species	% of total
Pseudomonas spp. (unidentified)	33.33
Aeromonas sobria	21.43
Pseudomonas putida	19.05
Pseudomonas cepacia	9.52
Pseudomonas fluorescens	9.52
Pseudomonas luteola	4.76
Aeromonas hydrophila	2.38

* For the identification of these organisms the appropriate computer software from the API company was used. The software is more appropriate for the identification of non-enteric organisms isolated from hospital sources.

Table 4.14. Test substrate, reactions and percentage of positive results of 42 bacteria isolated on *Pseudomonas* agar (Adams & Mead 1977) and tested with the API 20NE system

Test	Substrate	Kind of reaction	% positive
1. NO_3	KNO_3	reduction of NO_3 to NO_2	38.8
2. TRP	tryptophane	production of indole	88.8
3. GLU	glucose	acid production	11.1
4. ADH	arginine	hydrolysis of arginine	88.8
5. URE	urea	urease	38.8
6. ESC	aesculin	hydrolysis (β-glucosidase)	10.0
7. GEL	gelatin	hydrolysis (protease)	22.2
8. PNPG	p-nitro/β-D-ga/ose	β-galactosidase	16.6
9. GLU	glucose	assimilation	100.0
10. ARA	arabinose	#	77.7
11. MNE	mannose	#	83.3
12. MAN	mannitol	#	61.1
13. NAG	N-acetyl-glucosamine	#	61.1
14. MAL	maltose	#	33.3
15. GNT	gluconic acid	#	94.4
16. CAP	capric acid	#	94.4
17. ADI	adipic acid	#	22.2
18. MLT	malic acid	#	100.0
19. CIT	citric acid	#	100.0
20. PAC	phenylacetic acid	#	50.0
21. OX	tetraphenyl-diamine oxidase of cytochrome C		100.0
22. TRI*	tributyrin	lipolysis	86.0

* : The tributyrin test was done on 75 cultures

\# : assimilation

Table 4.15. Percentage of lipolytic organisms isolated from olives

Substrate	Organism	No of organisms tested	% Positive for lipolysis
Victoria blue	*Pseudomonas* sp.	61	73.8
	Yeasts	18	33.3
	Lactic acid bacteria	21	9.5
Tributyrin	*Pseudomonas* sp.	75	86.0
	Yeasts	50	91.3

DISCUSSION

Until now the effect of the indigenous microbial flora on the preservation of olives or olive oil has not been considered to be a major factor affecting the quality of table olives or olive oil. It was only recently that Kiritsakis and Markakis (1987) suggested that improvements in storage conditions in order to diminish microbial contamination of olives before fermentation or olive oil extraction should be studied further.

With the exception of Pelagatti's work (1978-1980), the microbiology of olives in orchards has not attracted as much attention as that given to phytopathological aspects of the olive tree. The phytopathologists have provided extensive information concerning the occurrence and distribution of plant knot disease caused by *Pseudomonas savastanoi* (Pyrovolakis & Weiltzien 1970; Ercolani 1971; Ercolani 1991). The conclusion by Pyrovolakis & Weiltzien (1970) that the distribution of this organism was similar between healthy and diseased trees (in the Greek island of Crete) was in accord with that of Ercolani (1971) who studied material from four different areas in Italy. Ercolani has published a number (Ercolani 1971, 1978, 1979, 1983, 1985, 1991) of papers on the microbiology of olive leaves, with special emphasis on *Pseudomonas* spp., but he has not given any information about the microbiology of olive fruits.

In his recent study of the microbiology of olive leaves, Ercolani (1991) found that 51% of the isolates were *Pseudomonas syringae*, 6.7% *Xanthomonas campestis* and 3.9% *Pseudomonas fluorescens*. His most interesting observation was on the structure of the bacterial communities on leaves. He reported that bacterial communities on leaves of a given age at a given time during any one year displayed a common structure but differed significantly from those on the leaves of the same age at a different time or on the leaves of a given age at any time during any one year. A similar

conclusion has been drawn by Pelagatti (1978-1980). He observed that weather conditions, sampling site, use of insecticides, the meteorological conditions in a specific area, the different cultivars, the irrigation (some cultivars not irrigated) and even isolation techniques could cause variations in the incidence of the species isolated. The fact that the above mentioned factors could influence the endogenous flora of olives makes almost impossible to study all of them together in a short period of time. In my study the statistical analysis (ANOVA, one way) revealed that this was the case with our survey in Greece. The sampling area, the physiological stage of the olives and the season influenced the counts of the bacteria examined in this survey. It was notable that the second year of this survey I was unable to isolate high numbers of pseudomonads in the selective medium (Pseudomonas agar, supplemented with CFC) used in this study. This could be attributted either to the time of our survey or to the use of insecticides. Indeed the second survey was done early in October while the first survey occurred at the end of November. Moreover the second survey was done in different geographical area from the first one and insecticides had been recently used.

In my study an unidentified *Pseudomonas* sp., *Pseudomonas putida, Pseudomonas cepacia* and *Pseudomonas fluorescens* were found to be the dominant organisms among the bacteria isolated on Pseudomonas agar (Table 4.13). I did not identify any *Ps. savastanoi* because this organism was not included in the API software which I used for the identification of the microorganisms isolated from CFC medium. Moreover this organism was not in our collection so it could not be used as a reference to compare it with our unidentified strain. Even so, in general the results of my survey are in agreement with those of previous workers.

The bulk of knowledge concerning the microbiology of olives has been related to their fermentation (e.g Balatsouras 1966) - my study has shown

that lactic acid bacteria are common contaminants of olive fruit - or the microbiology of olives during storage before olive oil production (Verona & Vallegi 1949; Florenzano 1952). The results reported by these workers were focused on moulds and yeasts. They reported mainly on the biochemical characteristics especially lipases production of their isolates. In my study most of the pseudomonads were lipolytic as shown with Victoria Blue agar containing olive oil or tributyrin agar. The number of yeasts hydrolysing tributyrin was larger than those hydrolysing lipids in olive oil in Victoria Blue agar. Only a few of the lactic acid bacteria produced lipases. The fact that most of the pseudomonads and yeasts were lipolytic could be an important observation coming out from my results in regards to olive oil quality. Indeed the increase of rancidity, the main deteriorative change of olive oil during storage, is related to the hydrolysis of the glycerides. However more emphasis has been given to the hydrolysis of the olive oil glycerides while the fruit is still on the tree due to endogenous lipase enzymes, compared to the contribution on the hydrolysis of the lipolytic enzymes from bacteria, yeasts and moulds. In other words if the olives are stored before processing, and especially if the storage is unsatisfactory (eg. piling in thick layers where the fruit is heated by its own respiratory activity) bacteria, yeasts that may grow on the fruit can elaborate their own lipases. The combined effect of the endogenous and microbial lipases may result in considerable rise of the acidity of the oil to the detriment of its quality.

Among the yeasts isolated in my study *Saccharomyces* and *Candida* spp. were the most common isolates while *Aspergillus* and *Rhizopus* spp. were the most commonly occurring fungi. Pelagatti (1978-1980) has also reported that the above mentioned yeasts were found in his study in Italy. In particular he isolated *Saccharomyces (S. Bayanus, S. cerevisiae, S. chevalieri, S. fermentati, S. globosus, S. italicus, S. oleaceus, S.rouxii* etc), *Candida (C. olea, C. parapsilopsis, C. tenuis, C. utilis* etc), *Pichia (P. fermentans, P.*

pinus, P.polymorpha, P. terricola, P. vini etc) and *Torulopsis (T. candida, T. glabrata, T. holmi, T. magnoliae, T. stellata* etc). Similar findings have been reported by Balatsouras (1966) who studied Greek black olives in brine.

Lactobacillus plantarum and *Lactobacillus brevis* were identified among the lactic acid bacteria isolated in this study. These two organisms were identified with the API system (API software). The most recent work concerning the identification of lactic acid bacteria is that of Pelagatti (1978-1980). He also identified *L. plantarum* and *L. brevis* as the most frequent isolates among the lactic acid bacteria. He reported that *L. delbrueckii*, *L. fermentum* and *L. coryniformis* subs. *coryniformis* were isolated from few varieties only. In his study he used API system also.

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APPENDIX

Letters in Applied Microbiology 1990, **10**, 217–220 GWG/89

Phenolic extract from olives: inhibition of *Staphylococcus aureus*

G.J.E. NYCHAS*, SOULA C. TASSOU & R.G. BOARD *University of Bath, School of Biological Sciences, Claverton Down, Bath BA2 7AY, UK*

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The presence of olive extract in different media with or without glucose retarded staphylococcal growth and accentuated the secretion of protein into the media. Moreover the extract influenced the electrophoretic patterns of proteins secretion into the culture supernatant fluid.

The antimicrobial properties of natural substances such as spices and essential oils have been known for a long time and the commercial potential for their use discussed by Wilkins & Board (1989). Limited studies of six major phenolic compounds contained in ethyl acetate extracts of green olives (Fleming *et al.* 1969) have shown that these have antimicrobial properties. Moreover *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and fungi (Juven & Henis 1970) were found to be inhibited by the phenolic glycoside, oleuropein, and its derivitive, aglycone of oleuropein. As far as can be ascertained, the literature on food poisoning bacteria and phenolic compounds extracted from olives contains no references to *Staphylococcus aureus* and the effects of the phenolic extracts on exoprotein secretion by this organism has not been reported. This paper presents the results of an investigation on the effect of extracted phenolic compounds of olive fruits on growth and exoprotein by *Staph. aureus*.

Materials and Methods

ORGANISM

Staphylococcus aureus S-6 (NCTC 10657), predominantly a producer of *Staphylococcus* Enterotoxin B, was maintained on Plate Count agar (Lab M) slopes at 4°C and subcultured at weekly intervals.

GROWTH MEDIA

NZ amine medium (NZA) containing (g/l): N-Z amine A (Sheffield Chemical Co. Norwich, NY, USA), 40·0; yeast extract (Oxoid), 4·0; K_2HPO_4, 1; and Brain Heart Infusion (BHI) medium containing (g/l): BHI solids (produced by Lab M, Bury, Lancs., UK) 17·5; tryptose (Lab M) 10·0; NaCl 5·0; Na_2HPO_4 25·0, were used to study growth and toxin production. The media were sterilized by autoclaving (121°C for 15 min). In some experiments glucose, sterilized by filtration (0·2 μm; Millipore), was used to supplement media at a final concentration of 0·2% w/v.

EXPERIMENTAL PROCEDURE

A loopful of culture from a Plate Count agar slope was used to inoculate 100 ml of NZA medium without glucose in a screw-capped bottle (200 ml). After overnight incubation at 37°C, 20–40 μl aliquots of this culture were used to inoculate 2 × 50 ml volumes of growth media with or without glucose and with or without phenolic extract (5 ml of extract per 45 ml of broth) in bottles (50 ml). These cultures were incubated statically for 24 h at 37°C. At regular

* Corresponding author. Permanent address: Institute of Food Technology, S. Venizelou 1, Lycovrisi 14123, Athens, Greece.

Table 1. The effect of different media and phenolic compounds extracted from olives on growth and exoprotein formation by *Staphylococcus aureus* S-6

Treatment		Bacterial density (absorbance 600 nm)	Exoprotein (μg/ml)	
		Incubation time (h)		
Type of medium	Addition of olive extract	11·00	0	11·00
NZA*	+	0·208	12·5	152
	−	0·502	12·5	100
BHI†	+	0·040	92·0	150
	−	0·125	92·0	105

* N-Z amine A medium.
† Brain Heart Infusion medium.

intervals samples (2 ml) were removed aseptically from each culture bottle and used immediately for growth determination, the remainder of each sample was frozen at −20°C until subsequent estimation of exoprotein.

GROWTH

The turbitity of cells of *Staph. aureus* was determined by measuring the optical density at 600 nm using a SP6-550 UV/VIS Spectophotometer. Alternatively subsamples (0·15 ml) of cultures was used to fill six wells of a microtitre plate. Uninoculated wells were filled (0·2 ml) with sterile distilled water in order to prevent desiccation of the inoculated ones. The Dynatech MR600 microplate reader was used and the data analysed with a link BBC microcomputer.

EXPROTEIN

The method of Sedmak & Grossberg (1977) was used to determine the secretion of exoprotein using bovine serum albumin (Sigma) as standard.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

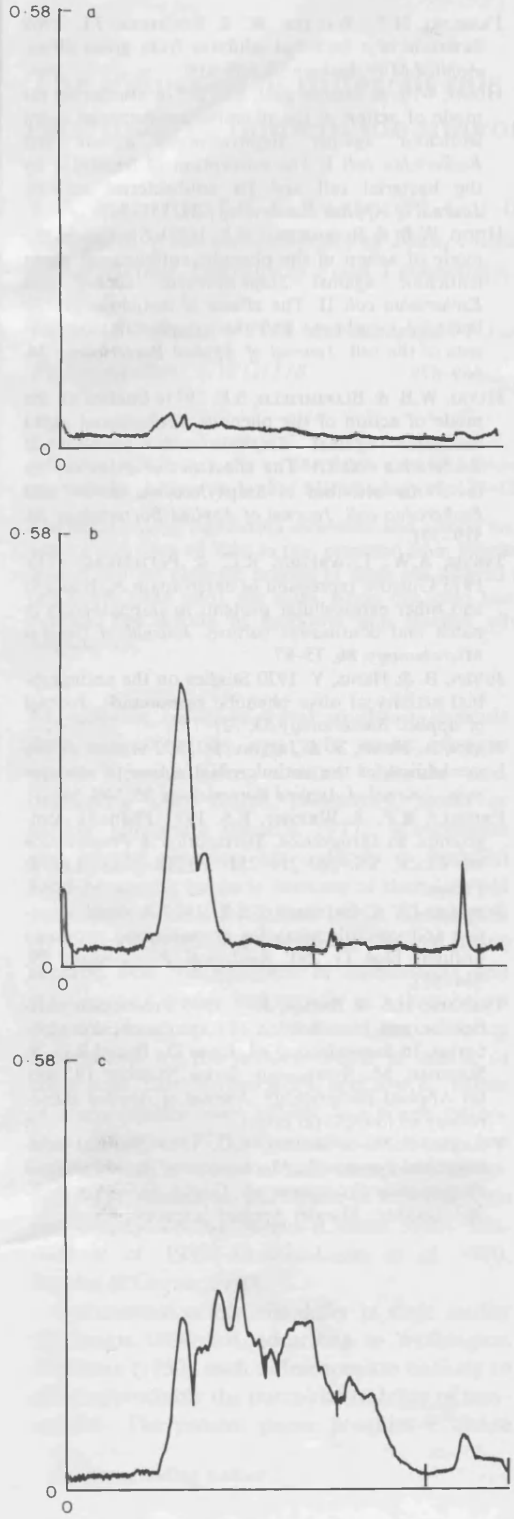
The protein profiles of uninoculated and inoculated growth media were determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis on gradient gels as described in the LKB laboratory manual for the Midget Electrophoresis Unit. Samples were freeze-dried (Morgan freeze-dryer, Modulyo). After staining (with 0·1% w/v Coomassie blue R-250 in 25% v/v methanol and 10% v/v acetic acid), gels were destained for 3 h in 40% (v/v) methanol and 10% (v/v) acetic acid. The destained gels were scanned (chromoscan 3, Joyce Loebl, Vickers plc) and traces obtained.

PHENOLIC EXTRACTS

Olive fruit (500 g) was mixed with 1 l water and brought to the boil. After a brief period of boiling, the aqueous extract was filtered through Whatman No 1 paper and the filtrate freeze-dried and kept at −20°C. Ten g of the freeze-dried olive powder were diluted in 60 ml of

Table 2. The effect of glucose and phenolic compounds extracted from olives on growth and exoprotein formation by *Staphylococcus aureus* S-6 in N-Z amine A medium

Treatment: addition of		Bacterial density (absorbance 600 nm)	Exoprotein (μg/ml)	
		Incubation Time (h)		
Extract	Glucose	9·30	0	9·30
+	−	0·120	12	52
+	+	0·420	12	32
−	−	0·490	12	47
−	+	0·680	12	39



water. This solution was extracted three to four times with petroleum-ether; these extracts were discarded. Extraction (three to four times) with ethyl acetate followed and the extract was concentrated with a rotary-evaporator. After all the ethyl acetate had evaporated, a yellow powder remained. Approximately 1 g was dissolved in 10 ml distilled water and used in this study.

Results

As would be expected from a previous study (Nychas, Tranter, Brehm & Board, unpublished data) the rate and the extent of growth of *Staph. aureus* in static cultures was markedly influenced by the composition of the growth medium. NZA medium was invariably superior to BHI when judged by cell proliferation and protein secretion. In practice, the most extensive growth was obtained with NZA medium supplemented with glucose (Tables 1 and 2). The presence of phenolic extract in NZA medium with or without glucose retarded staphylococcal growth but accentuated the secretion of protein into the medium. This feature, although less pronounced, was evident in unsupplemented BHI also. The presence of glucose in NZA medium accelerated *Staph. aureus* growth even in the presence of olive extract. It was notable, however, that olive extract in the presence of glucose depressed the extent of secretion of protein. The extract also influenced the pattern of protein secretion (Fig. 1). Indeed in this study two different electrophoretic patterns were obtained (Fig. 1b, c). Figure 1c shows the electrophoretic pattern of *Staph. aureus* grown without the addition of olive extract. The bands could not be attributed to protein degradation of the medium since no detectable proteolytic activity was present in a culture supernatant fluid after 11 h incubation (Nychas, unpublished observation). Moreover with NZA, an enzymatic digest of casein, there would be no protein other than those produced by staphylococci to serve as substrate for protease. When *Staph. aureus* was grown in the presence of olive

Fig. 1. Electrophoretic patterns of culture supernatant fluids during growth of *Staphylococcus aureus*; (a) uninoculated N-Z amine A medium; (b) with the addition of olive extract after 11 h of incubation; (c) without olive extract after 11 h of incubation.

extract two peaks were observed, which we attribute to the precipitation of extracellular or intracellular proteins of *Staph. aureus*.

Discussion

Many investigations have been concerned with the influence of BHI and NZA media on growth, toxin and exoprotein production by *Staph. aureus* (Transfer & Brehm 1990). In practice the rate of growth of *Staph. aureus* in NZA was always superior to that in BHI. The growth of this organism was accentuated by the addition of glucose whilst the exoprotein production was inhibited. These results are in accord with those reported by Jarvis *et al.* (1975) and Coleman *et al.* (1989). When phenolic extract was added to NZA (without added glucose), the extent of exoprotein formation increased. Previous investigations with phenolic and/or antioxidant compounds such as BHA and BHT, and those extracted with ethyl acetate from olives, indicate that these compounds cause leakage of constituents such as proteins, glutamate or potassium and phosphate from bacteria (Hugo & Bloomfield 1971a, b, c; Juven *et al.* 1972). This is due to enhanced membrane permeability. Prindle & Wright (1977) noted that the mode of action of phenols is concentration dependent. High amounts cause a precipitation of proteins in general whilst lower concentrations affect the activity of enzymes associated with energy production.

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The influence of different oils on the death rate of *Salmonella enteritidis* in homemade mayonnaise

S. A. RADFORD*, C.C. TASSOU*†, G.J.E. NYCHAS† & R. G. BOARD*‡ **School of Biological Sciences, University of Bath, Claverton Down, Bath BA2 7AY, UK and †Ministry of Agriculture, Institute of Food Technology, S. Venizelou 1, Lycovrisi 14123, Athens, Greece*

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The death rate of *Salmonella enteritidis* was always faster in mayonnaise made with extra virgin olive oil than in that prepared from blended olive or sunflower oils. The acidity and the phenolic profiles of these oils differed significantly. The most acidic oils (0·5% oleic acid), the extra virgin oils, also had the most complex phenolic profiles. The acidity of sunflower and blended olive oil was 0·2% and 0·4% respectively.

Mayonnaise, an oil-in-water emulsion, is made from vegetable oils, egg yolk, water, vinegar and/or lemon juice, sodium chloride and, depending upon recipe, sweeteners, spices or solium glutamate (Smittle 1977). Mayonnaise and related products are unlikely to transmit food-poisoning bacteria because of their low pH values, of about 4·1 (Collins 1985). Wethington & Fabian (1950) and Perales & Garcia (1990) showed that the numbers of salmonellas and staphlococci diminished rapidly at values below 4·1, the duration of the survival period being determined by the concentration of acetic or citric acid. Low temperature, and low a_w values in mayonnaises may inhibit foodborne pathogens (Smittle 1977). Products containing less than normal amounts of acid have been associated with foodborne outbreaks of salmonellosis and *Staphylococcus aureus* (Collins 1985; Mitchell *et al.* 1989; Gomez-Lucia *et al.* 1990; Perales & Garcia 1990).

Commercial edible oils differ in their acidity (Kiritsakis 1988) but, according to Wethington & Fabian (1950), such differences are unlikely to effect appreciably the microbial stability of mayonnaise. The present paper provides evidence that different grades of oil do influence the death rate of salmonellas in mayonnaise.

‡ Corresponding author.

Materials and Methods

MICROBIOLOGICAL ANALYSIS

Mayonnaise preparation

Mayonnaise (300 ml oil; 2 egg yolks (size 2); 9 ml (6% v/v) acetic acid; final pH 4·3) was prepared by whisking two egg yolks with an electric hand mixer (Carlton AMO3) and gradually adding the oil (150 ml) with a sterile pasteur pipette during continuous mixing. Six ml of acetic acid were whisked in to thin down the mixture so that it did not curdle. The remaining oil (150 ml) was then added and finally another 3 ml of acetic acid. The final pH, measured with a pH meter (EIL 7050), was 4·3. The following oils were used: sunflower, a proprietory blend of olive oils from EEC countries, and extra virgin olive oils from Italy and Greece.

Organism

Salmonella enteritidis strain PT4, kindly provided by Dr T. Humphrey (Exeter PHLS), was

maintained on Nutrient agar (lab m) slopes at 4°C and subcultured weekly. An overnight culture was grown in 50 ml nutrient broth (lab m) at 37°C, harvested by centrifugation, washed twice in saline and resuspended in saline to give a cell density of 10^9 cells/ml.

Inoculation

About 5×10^4 cfu/g *Salm. enteritidis* PT4 were throughly mixed with 40 g of mayonnaise by extensive stirring with a spatula. Uninoculated samples were used as controls. Samples were incubated at 20°C. The experiments were done four times with seven replicates per oil on one occasion and four in a further three trails.

Bacterial enumeration

At 0, 24, 48 and 72 h samples were diluted in 1/4 Ringer solution, 0·1 ml of an appropriate dilution was spread on nutrient agar in Petri dishes and colonies counted after 24 h at 37°C.

CHEMICAL ANALYSIS

Extraction of phenolic compounds

Oil (30·00 + 0·01 g) diluted in 50 ml hexane was extracted with 3 × 50 ml of methanol : water (60 : 40 *v/v*)—thorough mixing was achieved with a Kenwood chef mixer (Graciani Constante & Vazquez Roncero 1980). Two fractions were separated by centrifugation (300 × **g** for 10 min). The aqueous-alcoholic fractions from each extraction were filtered through damp Whatman No. 1 filter paper and evaporated under vacuum at 35–40°C. The residue was disolved in 2 ml methanol, filtered through a millipore filter (0·2 μm) and used for HPLC.

HPLC

The HPLC analyses were done by the method of Tassou *et al.* (1991). Oleuropein (Extrasynthex, France), and tyrosol, rutin, vanillic acid, caffeic acid and 3,4-dihyroxyphenylacetic acid (Aldrich Chemical Co. Ltd) were used for reference. All the standards were diluted in methanol and analysed by HPLC in the same way as those from the oils.

Determination of acidity

The method of Kiritsakis (1988) was used. An oil sample (28·2 g) in an Erlenmeyer flask (250 ml) was mixed with 50 ml ethanol (95% v/v) and 2 ml phenolphthalein (1% w/v in 95% ethanol) and titrated slowly with 1N NaOH until a pink colour appeared. The acidity was estimated thus: acidity (% oleic acid) = ml NaOH × Molarity NaOH × 0·282* × 100 × g^{-1} (*:0·282 = mg of oleic acid).

Results

MICROBIOLOGICAL ANALYSIS

The death rates of salmonellas in mayonnaise made with virgin olive oil (Greek or Italian) were faster than in those containing blended olive or sunflower oil (Table 1). No viable *Salm. enteritidis* were recovered from mayonnaise made with virgin oil after 72 h incubation but they were present in those made with blended or sunflower oil and incubated at 20°C for 3 d (Fig. 1). When all four experiments are considered (Table 1), the mayonnaise made from sunflower was always the least toxic. On two occasions the death rate of *Salm. enteritidis* in mayonnaise made from blended oil was not significantly different from that containing sunflower but on two occasions the former was faster than the latter. In all cases the death rates in mayonnaise made from extra virgin oil were significantly faster than those containing sunflower or blended oils.

Table 1. The effect of different vegetable oils on the death rate of *Salmonella enteritidis* PT4 in mayonnaise incubated at 20°C

	Experiment			
Oil used	1	2	3	4
F-test*	***	***	***	***
Sunflower	x†	x	x	x
Blend of olive oils	x	y	x	y
Italian olive oil (virgin)	y	ND	y	ND
Greek olive oil (virgin)	y	z	y	z

*** Significant at 0·1% probability level.

† Means with the same letter did not differ significantly. ND, Not determined.

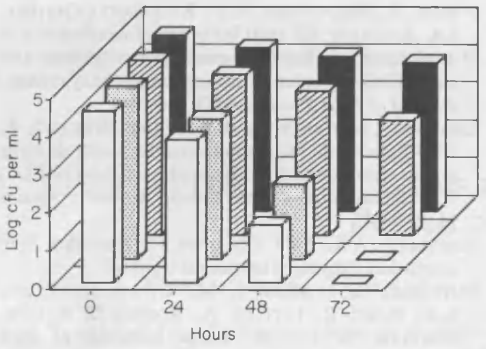

Fig. 1 The death rate of *Salmonella enteritidis* in mayonnaise made with different oils at 20°C. □, Italian extra virgin olive oil; ▨, Greek extra virgin olive oil; ▨, blended olive oil; ■, sunflower oil.

ANALYSIS OF OILS

There were significant differences ($P < 0{\cdot}1\%$) in the acidity of oils used—viz. sunflower (0·2%; least acid), blended olive oil (0·4%) and the two extra virgin oils (0·5%; most acid)—as well as their range of phenolic compounds. The blended olive oil and sunflower oil contained insignificant amounts of such compounds whereas the extra virgin olive oils contained the greatest range (Fig. 2). Tyrosol occurred at different concentrations (peak 3; Fig 2) and oleuropein (peak 10) only in the virgin olive oils, its concentration being greatest in that of Greek origin. This oil contained phenolic substances identified with caffeic acid (peak 4), rutin (peak 6) and vanillic acid (peak 7). Traces of protocatechuic acid (peak 2a) occurred only in the Italian virgin olive oil. Two unidentified peaks (2 and 11) were presented in the virgin olive oil of both origins. Traces of 3,4-dihydroxyphenylacetic acid (peak 2b) were identified only in the blended olive oil.

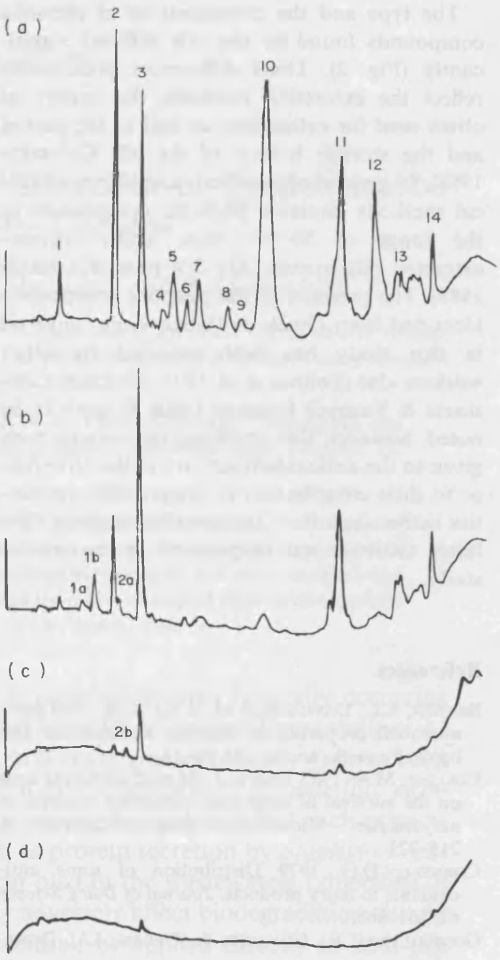

Fig. 2. The profiles of the phenolic compounds in (a) Greek extra virgin olive oil, (b) Italian extra virgin olive oil, (c) blended olive oil, and (d) sunflower oil. Peaks 2a, 2b, 3, 4, 6, 7 and 10 were identified with protocatechuic acid, 3,4-dihydroxyphenylacetic acid, tyrosol, caffeic acid, rutin, vanillic acid and oleuropein respectively; the rest of the peaks were unidentified.

Discussion

In systems such as mayonnaise there is a greatly expanded oil/water interface which exerts a marked influence on the distribution of long-chain free fatty acids and phenolics between the aqueous and oil phases (Cornell 1979; Collins 1985). It is well known, also, that long- and/or short-chain fatty acids as well as phenolic compounds act as antimicrobial agents on foodborne bacteria (Branen *et al.* 1980; Nychas *et al.* 1990; Tassou *et al.* 1991). These substances could well affect the fate of foodborne bacteria in mayonnaise. Edible oils contain both phenolic and long-chain fatty acids in various concentrations. The concentration of the long-chain fatty acids was the main difference between olive and sunflower oil. Although oleic (18 : 1) and linoleic (18 : 2) acids are present in both oils, the former is dominant in olive and the latter in sunflower oil (Kiritsakis 1988). In this study the acidity (free fatty acids) in the two virgin olive oils was higher than in the sunflower oil.

The type and the concentration of phenolic compounds found in the oils differed significantly (Fig. 2). These differences presumably reflect the extraction methods, the variety of olives used for extractions as well as the period and the storage history of the oil (Kiritsakis 1988). In general olive oil extracted by mechanical methods contains phenolic compounds in the range of 50–157 ppm whilst solvent-extracted oils contain 321–574 ppm (Kiritsakis 1988). The presence of the phenolic compounds identified from Greek or Italian virgin olive oil in this study has been reported by other workers also (Solinas *et al.* 1975; Graciani Constante & Vazquez Roncero 1980). It needs to be noted, however, that emphasis has usually been given to the antioxidant activity of the olive oils, or to their contribution to organoleptic properties rather than their antimicrobial activity. The latter attribute was emphasized in the present study.

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Effect of Phenolic Compounds and Oleuropein on the Germination of *Bacillus cereus* T Spores

CHRYSOULA C. TASSOU,* G. J. E. NYCHAS,* AND R. G. BOARD†

**Institute of Food Technology, Ministry of Agriculture, S. Venizelou 1, Lycovrisi 14123, Athens, Greece, and †School of Biological Sciences, University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom*

TASSOU, C. C., NYCHAS, G. J. E., AND BOARD, R. G. Effect of Phenolic Compounds and Oleuropein on the Germination of *Bacillus cereus* T Spores. *Biotechnol. Appl. Biochem.* **13,** 231–237 (1991).

The phenolic compounds extracted from olives with ethyl acetate inhibited germination and outgrowth of *Bacillus cereus* T spores. Purified oleuropein, a well-characterized component of olive extract, inhibited these processes also. The addition of oleuropein and olive extracts 3 or 5 min after germination began, immediately decreased the rate of change of phase bright to phase dark spores and delayed significantly outgrowth.

Studies of the fermentation of olives (1, 2) have shown that naturally occurring antimicrobial substances need to be removed in order to initiate fermentation. At least six major phenolic components of extracts from green olives (3, 4) have been associated with the inhibition of the fermentative organisms, *Lactobacillus plantarum* and *Leuconostoc mesenteroides.* Other studies have shown that such extracts inhibit microfungi and bacteria (5, 6) as well as protein secretion by *Staphylococcus aureus* (7). These inhibitory substances occur also in the wastewaters (alpechin) of olive oil mills (8). Indeed their presence may adversely affect biodegradation should such wastes, notable for their pollution potential, be applied directly to land (9). Microbiological studies have shown that the addition of waste waters to soil displaces susceptible members of the bacterial flora (10) especially aerobic spore forming bacteria (10, 11). In a recent study Rodriguez *et al.* (12), who worked with *Bacillus megaterium,* found that wastewaters from olive processing mills inhibited growth of vegetative cells as well as the germination and sporulation processes. The present study was concerned with spore germination and outgrowth of *Bacillus cereus* T, an organism that has been used in innumerable studies of many facets of spore physiology (13, 14). The main objective was to establish the phase of germination–outgrowth that was particularly susceptible to inhibition.

MATERIALS AND METHODS

Organism

Bacillus cereus T was maintained on potato dextrose agar (PDA;[1] pH 7.2, lab m code lab 98) slopes at 4°C and subcultured at weekly intervals.

[1] Abbreviations used: PDA, potato dextrose agar; TSB, tryptone soya broth; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

0885-4513/91 $3.00

Production and Cleaning of Spores

The method of Gould (15) was used. *Bacillus cereus* was grown at 30°C on PDA (pH 7.2). When sporulation was complete and the sporangia had lysed, the spores were washed off the agar with ice-cold distilled water, washed a further six times, and harvested by repeated centrifugation (4000*g*). Suspensions were cleaned of vegetative cells and debris by discarding the uppermost layers of the pellets obtained by centrifugation. The clean spore suspensions were stored at −20°C.

Extraction of Phenolic Compounds

Olives (200 g) were frozen in liquid nitrogen and, after removal of the stones, crushed in a mixer (Kenwood chef). The mixture was immediately homogenized in 80% (v/v) ethanol in water. After agitation at 4°C for 20 min followed by filtration (Whatman No. 1), the residue was again extracted with 80% ethanol. Aqueous–alcohol extracts were collected and ethanol was evaporated under vacuum. Four successive petroleum ether extractions removed pigments and most of the lipids. The phenolic compounds were then extracted with ethyl acetate. After three successive extractions, the ethyl acetate was removed under vacuum and the dry residue (0.5 g) dissolved in 10 ml water (16). The final extract was used in experiments with *B. cereus* spores. Part of the residue was dissolved in methanol and used for HPLC analysis.

HPLC Analysis

The chromatographic profile of the phenolic extract as well as the purity of oleuropein (Extrasynthese, 69730 Genay, France) were checked by HPLC. The analyses were carried out with an LCD Milton Roy high-pressure liquid chromatograph consisting of: two Model Constametric pumps, a Rheodyne 7125 injector, a Model 1204 A spectromonitor variable-wavelength detector set at 280 nm, a Chromatograph Control Module microprocessor, and a Model S-201 GP printer. An FSA 25 cm APEX C8 (particle size, 10 μm) column was used for reverse-phase analysis and a solution of acetonitrile/H_2O, adjusted to pH 2.6 with orthophosphoric acid, was used as the mobile phase (16). The program was run isocratically with 5% acetonitrile in water for the first 2 min, the concentration being increased to 18% at 10 min, 21% at 20 min, 27% at 22 min, and 54% at 35 min. The solvents were HPLC grade. The flow rate was 4 ml/min, and the injection volume 10 μl.

Effect of Oleuropein and Phenolic Extract from Olives on Germination of Bacillus cereus

A spore suspension (0.02 ml; 10^9–10^{10} cfu), was diluted in 3 ml of phosphate buffer (Na_2HPO_4–KH_2PO_4; pH 6.8) and activated by heating in a water bath at 70°C for 30 min. The spores were then separated from the phosphate buffer by centrifugation (4000*g*) and added to tryptone soya broth (TSB; lab m code lab 4). L-Alanine and inosine were added in final concentrations of 10 and 1 mM, respectively, and the optical density (OD) at 580 nm was measured with a Philips Pye Unicam PU 8650 spectrophotometer in a room at 30°C. Immediately and at regular intervals after inoculation, a 0.1-ml sample was removed from the cuvette and mixed with 0.4 ml of formaldehyde–saline (2% w/v) to arrest spore germination and outgrowth. A drop of

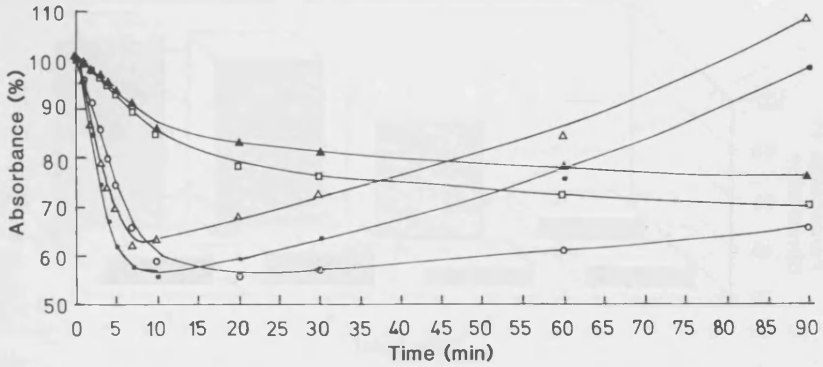

FIG. 1. The effect of different concentrations of oleuropein on spore germination and outgrowth of *B. cereus* T (closed circles, control; open triangles, 0.05%; open circles, 0.4%; open squares, 0.8%; closed triangles, 1%) (each point average of eight experiments).

this suspension was placed on a clean microscope slide, viewed with a phase-contrast microscope, and photographed. Three hundred spores, present in 10 randomly selected fields of view, were scored as phase bright or dark and the ratio of germinated (dark) to ungerminated (bright) spores expressed as a percentage. Various amounts of oleuropein or phenolic extract from olives were added at different times to a spore suspension of *B. cereus* in TSB. The phase bright to phase dark change of spores during germination was detected by measuring the fall in optical density. Because of changes due to oxidation of colored extracts, TSB with addition of the same amounts of the extracts was used as the control to monitor color changes. At the end of the changes of the optical density (about 90 min from the beginning of germination), decimal dilutions made from the samples in the cuvettes were inoculated on PDA (pH 7.2) in petri dishes. After a 24-h incubation at 30°C, the numbers of colonies were counted.

RESULTS

Inhibition of Spore Germination

Oleuropein. Figure 1 (the average results of eight separate experiments) shows the effects of different concentrations of oleuropein (80% pure according to the manufacturer) on the germination of *B. cereus* T spores in TSB at 30°C. In unsupplemented TSB, the changes from phase bright to phase dark spores occurred within 7 min. This was followed by rapid outgrowth of vegetative cells. These events were confirmed microscopically. TSB supplemented with 0.05% (w/v) oleuropein did not influence appreciably the rate of change from phase bright to phase dark but terminated this process at an earlier stage vis à vis the control. This concentration had no evident effect on subsequent outgrowth as shown in microscopic studies. An oleuropein concentration of 0.4% did not affect appreciably the phase bright to phase dark change but outgrowth was delayed markedly. Again this feature was confirmed microscopically. Higher concentrations of oleuropein (0.8 and 1% (w/v)) delayed both the rate and extent of phase change. Indeed microscopic examinations with 1% oleuropein (Fig. 2) revealed that the vast majority of spores remained phase bright. When

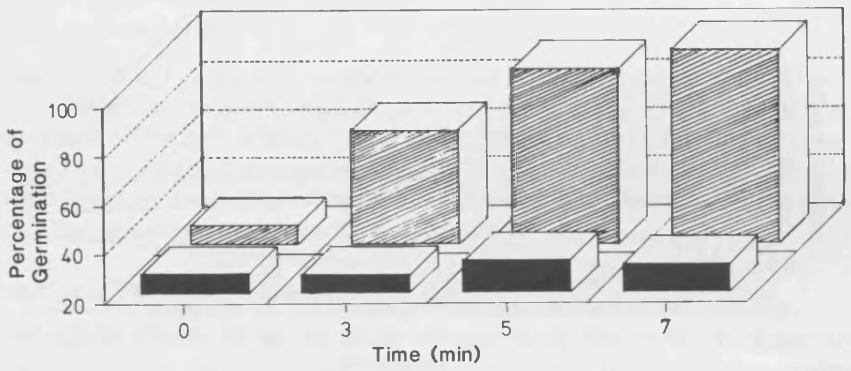

FIG. 2. The effect of 1% oleuropein on the percentage of phase dark spores during germination of *B. cereus* T (closed bars, 1%; hatched bars, control).

1% oleuropein was added 3 min after the initiation of germination, the rapid change of spores from phase bright to phase dark was arrested and there was no subsequent outgrowth (Fig. 3). When the same concentration was added at 5 min, almost at the end of the phase bright to phase dark change, the germinated spores again failed to proceed to outgrowth (Fig. 3).

Olive extract. Figure 4 shows the effect of different concentrations of olive extract on the germination of spores of *B. cereus* T. Addition of 1% (v/v) olive extract in the TSB spore suspension delayed both the rate and extent of change from phase bright to phase dark spores. Thus the decline in OD was arrested at an earlier stage than in the controls and subsequent outgrowth was delayed. Higher concentrations (2 and 4%) of olive extract progressively decreased the rate of phase change of spores and inhibited completely cell outgrowth. Figure 5 shows the effect of 4% olive extract when added 3 and 5 min after the beginning of germination. Addition after 3 min decreased slightly

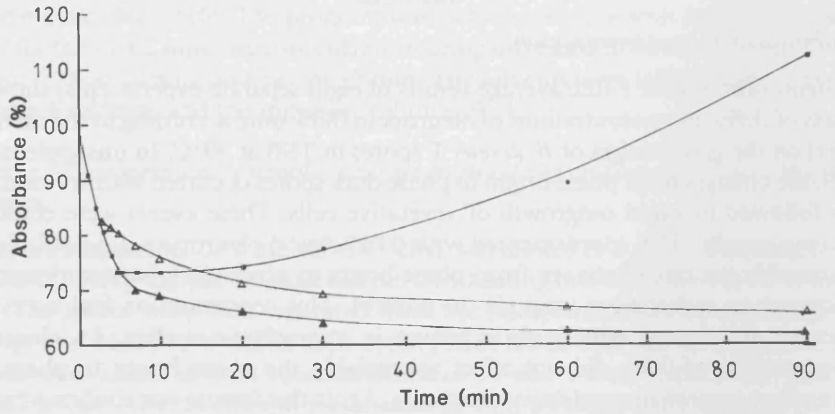

FIG. 3. The effect of the addition 1% oleuropein at various times in the germination process of *B. cereus* (closed circles, control; open triangles, at 3 min; closed triangles, at 5 min) (each point average of five experiments).

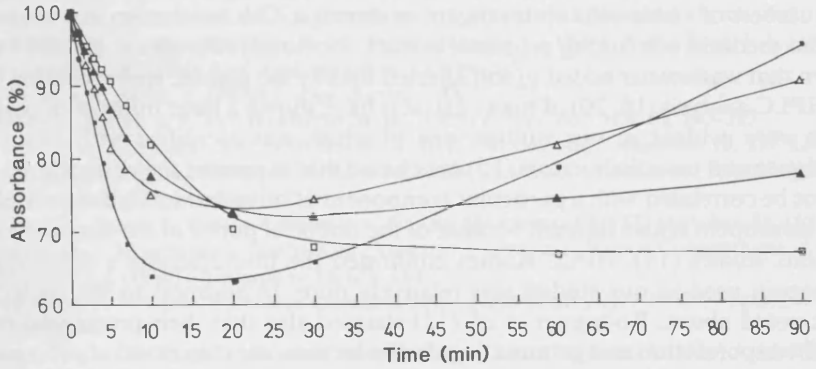

FIG. 4. The effect of different concentrations of olive extract on spore germination of *B. cereus* T (closed circles, control; open triangles, 1%; closed triangles, 2%; open squares, 4%) (each point average of six experiments).

the rate of change of spores from phase bright to phase dark but delayed outgrowth significantly. The latter event was also inhibited markedly when the extract was added 5 min after the initiation of spore germination. At this time the majority of the spores were phase dark.

DISCUSSION

The occurrence of phenolic compounds in olive fruits has been demonstrated by many workers using a variety of solvents (2, 17, 18). In our study we used the method of Amiot *et al.* (16), ethanol extraction followed by ethyl acetate extraction. Such extracts have been shown to inhibit a range of microfungi and bacteria (5, 17, 19). Rodriguez *et al.* (12) reported that an *n*-propanol extract of waste water from olive oil mills inhibited the growth of *B. megaterium* as judged by zones of inhibition around filter paper discs impregnated with the extract and placed on the surface of a nutrient agar seeded with the test organism. They also observed a decline of ca. 1 log cycle in

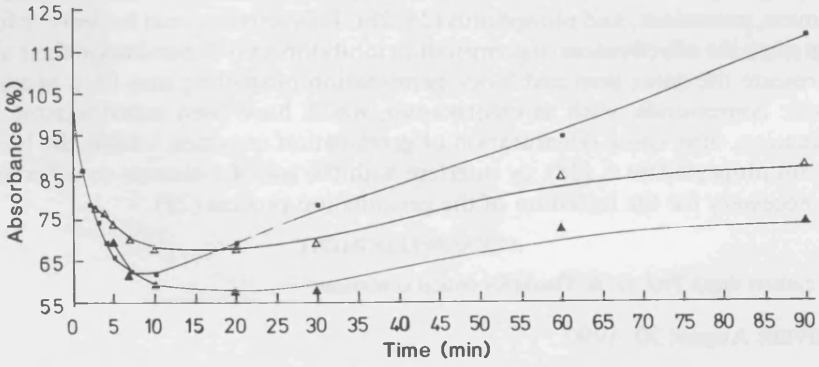

FIG. 5. The effect of 4% olive extract at various times during germination process of *B. cereus* T (closed circles, control; open triangles, at 3 min; closed triangles, at 5 min) (each point average of four experiments).

the numbers of viable cells of this organism during a 72-h incubation in a chemically defined medium containing propanol extract. Previously, Paredes *et al.* (10, 11) had shown that wastewater added to soil affected mainly the aerobic spore forming bacteria. HPLC analysis (16, 20) of many extracts have shown a large number of peaks; 41 peaks were evident in our studies, one of which was identified with oleuropein. Rodriguez and his collaborators (12) concluded that at present the antibacterial effect cannot be correlated with a particular component of olive extract. Indeed correlation with oleuropein is also difficult because of the doubtful purity of the material used in previous studies (19). HPLC studies confirmed the manufacturer's claim that the oleuropein used in our studies was relatively pure. In addition to the bactericidal effect noted above, Rodriguez *et al.* (12) claimed also that their propanolic extract inhibited sporulation and germination. In the former case they noted about a half-log decrease during an 8-h incubation of a mineral medium seeded with spores collected from a 3-day-old culture. When propanolic extract was present, there was no change in spore counts. The counts were done with a Petroff–Hausser chamber but apparently no effort was made to distinguish between phase bright and phase dark spores. Inhibition of sporulation was judged by doing viable counts on pasteurized (80°C for 10 min) 3-day-old culture in medium with or without propanolic extract. In the first instance 90% of the counts were attributed to spores whereas in the second the proportion was reduced to 45%. Whether or not the latter were remnants from the inoculum is not clear. Our studies were focused on the action of extract of olive fruit as well as of oleuropein on the phase change and outgrowth of spores of *B. cereus* T. It was evident that at effective concentrations the supplements affected both spore germination and subsequent outgrowth (Figs. 1 and 4). The inhibition, therefore, appears at the very primary stage (transformation of the phase bright spore to a phase dark form) of the development process from spore to vegetative cell (Figs. 1–4). Moreover, it was found that the addition of oleuropein and olive extract at various times during the process of germination inhibited the outgrowth of the germinated spores (Figs. 3 and 5). It is well known that during outgrowth the principal changes occurring are the initiation of RNA, protein, and membrane syntheses (21). It has been found that the addition of phenols to bacteria, sporulated or not, affected either membrane synthesis or inactivated cellular enzymes (22–24). Indeed it was found that phenols such as BHA, BHT, and oleuropein attack the cytoplasmic membrane, destroying its permeability and causing leakage of intracellular constituents such as glutamate, potassium, and phosphorus (25, 26). This activity could be decisive for the spores since the effectiveness of germination inhibitors may depend upon their ability to permeate the spore coat and block germination-promoting sites (27). Moreover, phenolic compounds, such as chlorocresol, which have been tested against spore germination, may cause denaturation of germination enzymes, inhibit the lytic enzyme subtilopeptidase A (28), or interfere with the use of L-alanine or other amino acids necessary for the initiation of the germination process (29).

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The effect of the olive phenolic compound, oleuropein, on growth and enterotoxin B production by *Staphylococcus aureus*

H.S. Tranter, Soula C. Tassou[1] and G.J. Nychas[1]

Division of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts, UK and [1]National Agricultural Research Foundation, Institute of Food Technology, S. Venizelou 1, Lycovrisi, Athens, Greece

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H.S. TRANTER, S.C. TASSOU AND G.J. NYCHAS. 1993. The presence of low concentrations (0·1% w/v) of oleuropein, a phenolic compound extracted from olives, delayed the growth of *Staphylococcus aureus* in NZ amine A and brain heart infusion media modified by the addition of growth factors and glucose (NZA + and BHI +), as indicated by changes in conductance, whilst higher concentrations (0·4–0·6% w/v) inhibited growth completely. Intermediate concentrations of oleuropein (0·2%) prevented growth in BHI + but allowed growth to occur in NZA + despite an extended lag phase (30 h). Concentrations of oleuropein > 0·2% inhibited growth and production of enterotoxin B in both types of media. Lower levels (0·1%) did not affect the final viable count and production of toxin in BHI + but decreased the number of viable organisms and reduced the toxin production in NZA + by eightfold. An increase in the concentration of oleuropein resulted in a decrease in the amount of glucose assimilated and consequently the amount of lactate produced. In addition, oleuropein prevented the secretion of a number of exoproteins. Addition of oleuropein during the exponential phase appeared to have no effect on the growth of *Staph. aureus* in NZA +.

INTRODUCTION

The inhibition of fermentative organisms such as *Lactobacillus* spp. during olive fruit fermentation has been associated with at least six major phenolic compounds which may be extracted from green olives by ethyl acetate (Fleming *et al.* 1969, 1973). These compounds include the phenolic glycoside, oleuropein, and its hydrolysis products β-3,4-dihydroxyphenylethyl alcohol, elenolic acid and oleuropein aglycone. Such extracts have been shown to inhibit the growth of a number of Gram-negative bacteria (Fleming *et al.* 1973; Garrido-Fernandez and Vaughn 1978) and some fungi (Gourama and Bullerman 1987). In addition, Tassou *et al.* (1990) showed that oleuropein was able to prevent the germination of *Bacillus cereus* spores. Indeed the presence of such inhibitory compounds in the waste waters (alpechin) of olive oil mills may affect the microflora, especially the aerobic spore-forming bacteria, of soils treated with such waste material (Vazquez-Roncero *et al.* 1974).

The antimicrobial activity of many naturally occurring compounds has been reviewed (Paster *et al.* 1988; Wilkins and Board 1989) and there is considerable interest from the food industry in the use of these compounds as alternative food additives to prevent or delay the growth of food pathogens or the onset of food spoilage. Nychas *et al.* (1991) showed that oleuropein was able to inhibit protein secretion by *Staph. aureus* but there have been no other studies on the effect of phenolic extracts of olives on food poisoning bacteria or production of bacterial toxins. This study was initiated to examine the effect of oleuropein on the growth of and production of enterotoxin B by *Staph. aureus*. In addition, the measurement of changes in the conductance of growth media was used as a convenient and rapid means of studying the effect of antimicrobial compounds on bacterial growth.

Correspondence to: Dr H.S. Tranter, Division of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts SP4 0JG, UK.

MATERIALS AND METHODS

Bacterial strain

Staphylococcus aureus S-6 (NCTC 10657), which produces enterotoxin B and small amounts of enterotoxin A, was used. The culture was maintained on slopes of Plate Count agar at 4°C and subcultured every 4–6 weeks.

Growth media

NZ amine A medium (NZA) (containing (g l^{-1}): NZ amine A (Sheffield Chemical Co., Norwich, NY, USA), 20·0; proteose peptone No. 3 (Difco), 20·0 and Brain Heart Infusion broth (BHI; Oxoid) were sterilized by autoclaving (121°C for 20 min). Filter-sterilized (Millex, 0·02 μm; Millipore) growth factor solution (1% w/v niacin and 0·5% w/v thiamin) was added to each medium to give final concentrations of 0·001% (w/v) niacin and 0·0005% (w/v) thiamin, respectively and 1% (w/v; final concentration) glucose, sterilized by autoclaving (121°C for 20 min), was added immediately prior to inoculation (NZA+ and BHI+, respectively).

Experimental procedure

The growth of bacteria was monitored by the Malthus 2000 (Radiometer International, Copenhagen), a commercially available microbiological analyser. The instrument consists of constant temperature incubators that hold reaction cells containing the microbial cultures and electrodes. Any micro-organism present in test samples and able to grow in the medium in the reaction vessel begins to metabolize, producing changes in the conductance of the growth medium. The analyser monitors conductance in the reaction vessels every 6 min and records the data; any significant increase in conductance (approximately 8 μs) from a baseline in the test samples is recorded as a detection time which will vary according to the number of micro-organisms originally present in the sample.

Growth medium (NZA+ or BHI+) was dispensed (2·5 ml final volume) into sterile (121°C/20 min) closed glass reaction cells containing platinum electrodes (Radiometer Co., Denmark). Oleuropein (Extrasynthase, Genay, France) was dissolved in distilled water (250 mg ml^{-1}). The suspension was warmed to 40–50°C to aid dissolution and the solution sterilized by filtration (Millex, 0·02 μm; Millipore). This solution was added to duplicate reaction cells each containing growth medium (NZA+ or BHI+) to give a final concentration of 0–0·6% (w/v) oleuropein in a final reaction mixture volume of 2·5 ml. These reaction tubes were incubated at 37°C for 1 h before inoculation with 0·1 ml of an 18 h culture of *Staph. aureus* S-6 grown at 37°C in the same growth media. After inoculation the reaction tubes were incubated in the water bath of a Malthus 2000 system maintained at 37°C and the electrodes connected to the analyser. After 48 h samples (1 ml) were removed aseptically from the reaction cells for determination of both the number of viable organisms and enterotoxin concentration.

In some experiments the effect of oleuropein on organisms that were growing in media to which oleuropein had not been present initially was studied. This was done by the addition of 50 μl of sterile oleuropein (0·1–0·4% w/v, final concentration) or distilled water to reaction mixtures 6 h after the inoculation of 2·5 ml of medium with 0·2 ml of an overnight (18 h) culture of *Staph. aureus* S-6.

Viable count

Culture samples (1 ml) were serially diluted in 9 ml amounts of Ringer solution. Six 0·02 ml amounts of each solution were placed individually on the dried surface of Plate Count agar (Lab M) and the colonies counted after 24 h at 37°C.

Enterotoxin assay

The production of enterotoxin B in the presence or absence of oleuropein was estimated by the reversed passive latex agglutination kit (Unipath) according to the manufacturer's instructions. A sample (1 ml) was removed from the reaction cells, centrifuged (10 000 *g*) for 5 min to remove the organisms and the supernatant fluid removed and stored at −80°C. The toxin present in culture fluids was expressed as the reciprocal of the last dilution which gave an agglutination reaction.

Glucose and lactic acid determination

Concentrations of L-lactate and glucose in samples of supernatant fluids were estimated by the lactate dehydrogenase reaction (Gutman and Wahlefeld 1974) and the GOD-Perid kit (Boehringer, Mannheim), respectively.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protein profiles of uninoculated and inoculated growth media were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) on gradient gels (Nychas and Board 1991). The destained gels were scanned with a scanning densitometer (S.A. Helena, France).

RESULTS

Comparison of growth media

Typical conductance curves obtained during growth of *Staph. aureus* S-6 in NZA+ and BHI+ media are shown in Figs 1 and 2, respectively. Although the detection time (2·2 h for NZA+ and 2·7 h for BHI+) and conductance changes were similar in both media, the increase in conduc-

tivity in NZA + medium was almost twice that observed in BHI + medium. This increased conductivity did not appear to be due to larger numbers of organisms as the viable counts in both media after 48 h were almost identical (Table 1). Initially there was a rapid change in conductivity corresponding to the exponential growth phase followed by a second much slower increase in conductivity which was greater in BHI + media than NZA +.

Effect of oleuropein on growth

Oleuropein inhibited growth of *Staph. aureus* S-6 in both types of media as indicated by an increase in both the lag phase and exponential phases of growth. There was a clear relationship between the extent of inhibition and the concentration of oleuropein (Figs 1 and 2). Growth was completely inhibited by higher concentrations of oleuropein (>0·4%) in both media whereas lower concentrations (0·1%) delayed the onset of and slowed the rate of growth of the organism (Figs 1 and 2 and Table 1). An intermediate concentration (0·2%) completely inhibited the growth of *Staph. aureus* in BHI + media but allowed growth to occur in NZA + medium despite a long delay in the lag period (approximately 30 h). The Malthus 2000 system recorded extended detection times for organisms in both media in the presence of higher concentrations of oleuropein (Table 1) and, although there was a small increase in the conductivity (approximately 50 μS) of these samples over 48 h (Figs 1 and 2), there was clearly no observable

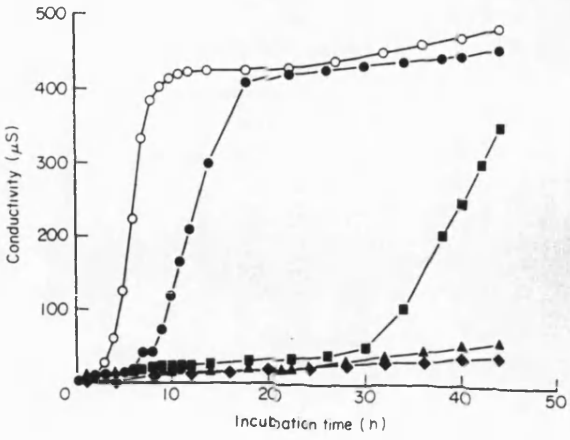

Fig. 1 Conductance curves obtained during growth of *Staphylococcus aureus* S-6 in NZA + medium in the presence of oleuropein. ○, Medium only; ●, medium + 0·1% oleuropein; ■, medium + 0·2% oleuropein; ▲, medium + 0·4% oleuropein; ◆, medium + 0·6% oleuropein

growth in these tubes. As long detection times were occasionally recorded in uninoculated medium such observations may have been the result of small fluctuations in the medium baseline which the Malthus registers as a detection time. Although the presence of oleuropein in media at the time of inoculation delayed the onset and slowed the rate of growth of *Staph. aureus* S-6, addition

Table 1 Growth of *Staphylococcus aureus* S-6 in NZA + and BHI + and the effect of oleuropein on detection time, glucose utilization and production of lactate and enterotoxin B after 48 h at 37°C

Growth medium	Oleuropein concentration (% w/v)	Detection time (h)	Viable count per 2·5 ml*	Enterotoxin B RPLA titre†	Glucose concentration (mg 100 ml^{-1})‡	Lactate production (mg 100 ml^{-1})§
NZA +	—	2·2	$9·1 \times 10^8$	64 000	26·5	0·82
NZA +	0·1	7·0	$6·5 \times 10^8$	8000	57·8	0·72
NZA +	0·2	30·8	$5·3 \times 10^8$	200	278·0	0·27
NZA +	0·4	46·5	< 500	100	468·0	0·19
NZA +	0·6	46·5	< 500	100	680·0	0·20
BHI +	—	2·7	$7·8 \times 10^8$	64 000	15·0	1·39
BHI +	0·1	1·5	$1·2 \times 10^9$	64 000	64·0	1·53
BHI +	0·2	46·5	$1·3 \times 10^5$	800	261·0	0·25
BHI +	0·4	46·5	< 500	400	310·0	0·25
BHI +	0·6	23·0	< 500	400	925·0	0·25

* Initial inoculum level in NZA + = 6×10^5 cells 2·5 ml^{-1} and BHI + = 3×10^6 cells 2·5 ml^{-1}.
† Reversed passive latex agglutination (RPLA) titre = reciprocal of last dilution of culture supernatant fluid giving agglutination.
‡ Initial glucose concentrations were estimated to be 1036·5 mg 100 ml^{-1} and 1153·2 mg 100 ml^{-1} in NZA + and BHI +, respectively.
§ Initial lactate concentrations were estimated to be 0·21 mg 100 ml^{-1} and 0·15 mg 100 ml^{-1} in NZA + and BHI +, respectively.
NZA +, NZ amine A medium plus growth factors and glucose; BHI +, brain heart infusion broth plus growth factors and glucose.

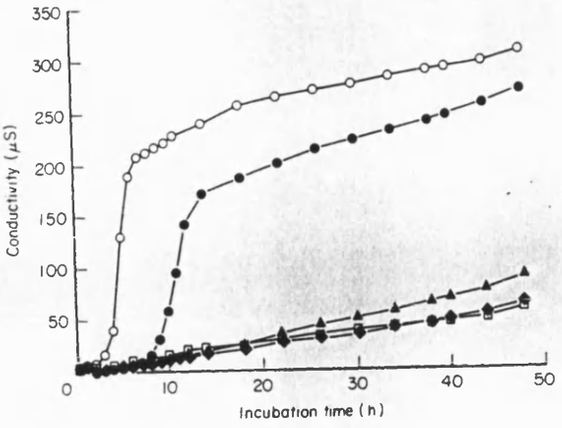

Fig. 2 Conductance curves obtained during growth of *Staphylococcus aureus* S-6 in BHI + medium in the presence of oleuropein. ○, Medium only; ●, medium + 0·1% oleuropein; □, medium + 0·2% oleuropein; ▲, medium + 0·4% oleuropein; ◆, medium + 0·6% oleuropein

of 0·1–0·4% (w/v) oleuropein midway through the exponential growth phase did not appear to affect growth in NZA + medium as demonstrated by changes in conductivity (Fig. 3).

Effect of oleuropein on toxin production

The effect of oleuropein on viable cell count and production of toxin in both growth media is shown in Table 1.

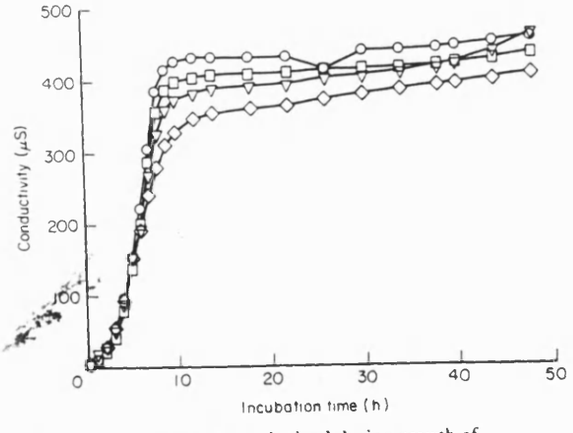

Fig. 3 Conductance curves obtained during growth of *Staphylococcus aureus* S-6 in NZA + medium showing the effect of adding 0·6% oleuropein after 6 h incubation. ○, Medium only; □, medium + 0·1% oleuropein; ▽, medium + 0·2% oleuropein; ◇, medium + 0·4% oleuropein

Despite an apparent difference in the rate of increase in conductance of NZA + medium compared with BHI + medium in the absence of oleuropein (Figs 1 and 2), the viable count at 48 h was similar and the toxin titre identical in both media. With NZA + medium an increase in the concentration of oleuropein in the medium led to an increased detection time with a decrease in the final (48 h) viable count and toxin production. A concentration of 0·2% (w/v) oleuropein was sufficient to inhibit growth such that the viable count was approximately half that obtained in the control culture without oleuropein while at the same time inhibiting toxin production by a factor of 320. Indeed, the low titres of toxin measured in cultures with >0·2% oleuropein may represent residual toxin carried over with the initial inoculum although this was not verified.

Although a similar pattern was obtained when *Staph. aureus* was grown in BHI + medium (Table 1), 0·1% (w/v) oleuropein appeared to give a small increase in the final viable count without affecting the toxin titre. When compared with a culture without oleuropein, this concentration appeared to result in a shorter lag phase on the basis of a reduced detection time (1·5 h; Table 1) but the full results show (Fig. 2) that the initial rapid phase of change in conductance did not begin until 8–9 h after inoculation.

Although the titres of toxin measured in BHI + medium containing higher (>0·2% w/v) concentrations of oleuropein appeared to be higher than those in NZA +, titres of toxin of approximately 100–400 may well represent toxin that had been carried over from the initial inoculum and net production of toxin in both types of growth media did not appear to occur in the presence of concentrations of oleuropein of 0·2% or higher.

Glucose utilization, lactate formation and exoprotein profiles

Glucose utilization and L-lactate formation were greatest in media (both NZA + and BHI +) that did not contain oleuropein (Table 1). In general, an increase in the concentration of oleuropein in both media reduced both glucose utilization and L-lactate formation by *Staph. aureus*. Analysis of growth media by SDS PAGE after 48 h showed the presence of a number of proteins in the molecular weight range 10–12 kDa. The presence of oleuropein in NZA + medium prevented accumulation of these proteins in the medium (Fig. 4). Similar results (not shown) were also observed with BHI + medium. The production of these proteins was not related to breakdown products of the medium itself as similar bands were not observed in uninoculated medium incubated for the same period. Furthermore, NZA, an enzymatic digest of casein, contains no

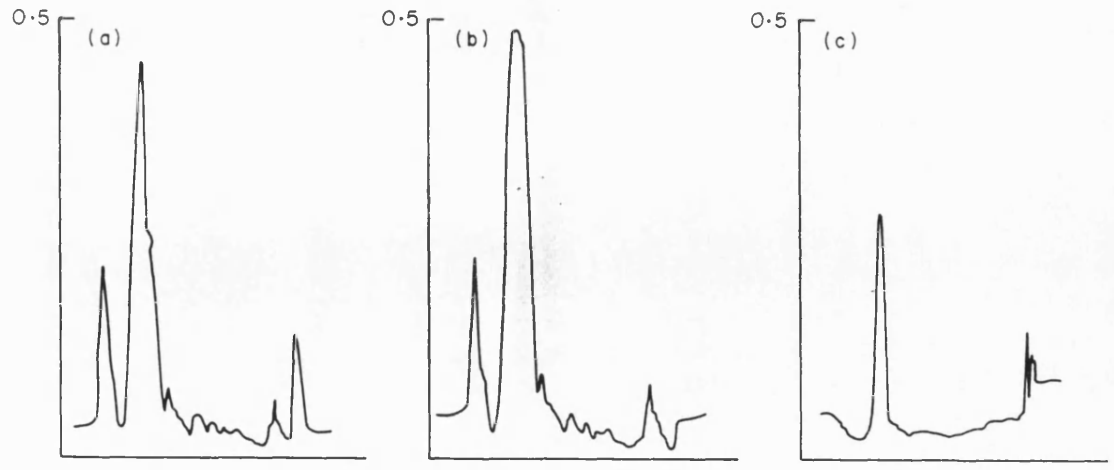

Fig. 4 Electrophoretic patterns of culture supernatant fluids after 48 h growth of *Staphylococcus aureus*. (a) NZA + medium only; (b) NZA + containing 1% oleuropein; (c) NZA + containing 0·4% oleuropein

proteins or peptides with molecular sizes >3000 Da (Nychas *et al.* 1991).

DISCUSSION

Changes in electrical conductance in microbiological media resulting from bacterial growth (Firstenberg-Eden and Eden 1984) can be measured by the Malthus 2000 system. During growth, uncharged or weakly charged substrates (proteins and carbohydrates) are converted into highly charged end-products (amino acids, lactate, etc.) resulting in increased conductance of the growth medium. In this study growth of *Staph. aureus* S-6 caused an increase in conductance in NZA + medium over the growth cycle which was almost twice that in BHI + medium. In a comparison of the two media Nychas *et al.* (1991) showed that while BHI contained higher amounts of proteins, NZA, a pancreatic digest of casein, contained higher concentrations of readily available nitrogen sources including amino acids, small peptides and ammonia. Such compounds probably contribute to the greater change in conductance observed with cultures grown in NZA +. Although the pattern of growth was similar in both types of media the initial growth phase after the lag phase in NZA medium lasted approximately 5 h and resulted in an increase in conductance of 400 μS compared with a growth phase after the lag phase of 2 h and a change in conductance of 200 μS in BHI medium. Despite these differences the final number of viable organisms in both media was similar.

The antimicrobial activity of oleuropein, a bitter glucoside present in the pulp of olive fruits, has been shown (Fleming and Etchells 1967; Juven *et al.* 1968; Ruiz-Barba *et al.* 1991), although others have been unable to demonstrate such activity (Fleming *et al.* 1973). Indeed, Garrido-Fernandez and Vaughn (1978) reported that oleuropein at concentrations of 0·2% (w/v) and 0·4% (w/v) could be utilized by micro-organisms associated with olive fermentations in basal medium but only after a delay in growth of 2–5 d. In this study low concentrations (0·1–0·2% w/v) of oleuropein delayed the onset of growth of *Staph. aureus* in NZA + medium by up to 30 h whilst higher concentrations (0·4–0·6% w/v) inhibited growth completely. Indeed growth did not occur even after incubation for 4 d in the presence of higher concentrations of oleuropein. Results in Table 1 suggest that higher concentrations of oleuropein may have been bactericidal to *Staph. aureus* resulting in a decrease in the number of viable organisms. In BHI + medium 0·1% (w/v) oleuropein delayed the onset of growth by approximately 4 h and higher concentrations inhibited growth totally. However, the addition of oleuropein during the exponential growth phase of the organism had little or no effect on growth in NZA medium. Phenolic and/or antioxidant compounds are known to cause leakage of cell constituents such as proteins, glutamate or potassium and phosphate from bacteria (Hugo and Bloomfield 1971a, b, c; Juven *et al.* 1972; Hugo 1991) which may be due to disruption of cell peptidoglycan (Ruiz-Barba *et al.* 1990) and/or damage of the cell membrane (Panizzi *et al.* 1960). Prindle and Wright (1977) indicated that the mode of action of phenolic compounds was concentration-dependent. High amounts precipitate proteins whilst lower concentrations affect the activity of enzymes associated with energy production. The differences observed with oleuropein treatment of non-growing compared with actively-

growing cells may be connected to the permeability of the cell outer layers during such times in the growth cycle.

Although oleuropein has been shown to stimulate growth of *Aspergillus* spp. with concomitant inhibition of production of aflatoxins B1, B2, G1 and G2 (Gourama and Bullerman 1987), there are no reports of the effect of this compound on bacterial exotoxin production. Nychas *et al.* (1990), however, clearly demonstrated that extracts from olives could inhibit growth and protein secretion from *Staph. aureus*. In this study while high concentrations of oleuropein inhibited growth of *Staph. aureus* S-6, low concentrations (0·1% w/v) were also shown to inhibit production of enterotoxin B in NZA+ medium although there was little or no effect on the final number of viable organisms. In BHI+ medium this same concentration (0·1% w/v) did not affect growth or toxin production from this strain, possibly because the higher protein concentration in this medium masked the antibacterial properties of these compounds (Ruiz-Barba and Jimenez-Diaz 1989).

Nychas *et al.* (1991) showed that addition of glucose to NZA and BHI medium resulted in a drop in pH due to the lactic acid produced during growth which may suppress production of enterotoxin B (Metzger *et al.* 1973). This effect can be eliminated during this study as the presence of oleuropein prevented the production of lactate by assimilation of glucose which consequently did not alter the pH of the growth media.

As the food industry searches for naturally-occurring antimicrobial compounds further investigations into the effect of phenolic compounds such as oleuropein on microorganisms should be carried out. The effect of oleuropein in combination with other antibacterial agents such as NaCl should be examined, particularly as the inhibitory effect of oleuropein appears to be accentuated in the presence of 5% NaCl (Fleming *et al.* 1973). Furthermore, it has recently been reported that oleuropein retains activity even after heating at 121°C for 15 min (Ruiz-Barba *et al.* 1991) which may not preclude its use in heat-processed foods. Automated conductance measurement using the Malthus 2000 provides a rapid and sensitive system for studying the effects of antimicrobial factors on toxin-producing bacteria. Many samples can be handled simultaneously allowing the screening of several antimicrobial compounds or single compounds over wide-ranging conditions. Care should be exercised, however, when organsims are cultured in growth media which are not specifically designed for the Malthus 2000. The apparent detection times recorded by the instrument during the incubation of sterile uninoculated media in this work may have resulted from fluctuations in the conductance baseline and may be a cause for concern during the detection of micro-organisms in food by this method. Microbiologists should be aware that solitary recordings of detection times by the Malthus instrument need not necessarily mean the presence of microbial activity and such samples should be continually incubated to establish further increases in conductance.

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Inhibition of Staphylococcus aureus by Olive Phenolics in Broth and In A Model Food System

C.C. Tassou and G. J. E. Nychas

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319 FIB, East Campus
University of Nebraska
Lincoln, NE 68583-0921 U.S.A.

INHIBITION OF *Staphylococcus aureus* BY OLIVE PHENOLICS IN BROTH AND IN A MODEL FOOD SYSTEM

Tassou C.C & Nychas G.J.E*

National Agricultural Research Foundation
Institute of Technology of
Agricultural Products
Sof. Venizelou 1, Lycovrisi 14123
Athens Greece
Tel; 301/2845940-2
fax; 301/2840740

Running Title: Inhibition of *Staphylococcus aureus*
Key words: *Staphylococcus*, inhibition, oleuropein, model food system, phenolics
* author for correspodence

ABSTRACT

The commercial 'pure' oleuropein and phenolics extracted from olives inhibited the growth and enterotoxin production by *Staphylococcus aureus* S-6 in broth as well as in reconstituted milk (model food system). It was found that the inhibition of this organism in N-Z Amine A broth was influenced by the initial inoculum size, the pH of the media and the concentration of additive. In particular growth and enterotoxin B production by *S.aureus* was inhibited in broth with a high concentration of oleuropein (0.6%). The inhibition was more pronounced in samples with low pH and low inoculum size. In the case of milk enterotoxin B production was also influenced by the initial concentration of extract.

Introduction

The antimicrobial activity of many naturally-occurring compounds has been reviewed (*37*) and there is considerable interest regarding the use of these compounds as alternative food additives to prevent or delay the growth of food pathogens or the onset of food spoilage. Among those chemicals which have been used and found to be safe are phenolic antioxidants which may have a potential preservative action in foods.

The occurrence of phenolic compounds in olive fruits and their potential antimicrobial action in different broth media have been demonstrated by many workers using a variety of solvents and test organisms (*6,7,17,36*). Extracts from olives have been shown to inhibit or delay the rate of growth of a range of bacteria and microfungi such as *Pseudomonas fluorescens, Lactobacillus plantarum, Bacillus megaterium, Staphylococcus aureus*, Enterobacteriaceae, *Aspergillus* spp., *Saccharomyces, Pichia, Torulopsis, Candida* (*7, 10, 12, 15, 16, 17, 20, 25, 26, 30, 33, 35*). These compounds include the phenolic glucoside, oleuropein, and its hydrolysis products β-3,4-dihydroxyphenylethyl alcohol, elenolic acid and oleuropein aglycon. In particular the suggestion that oleuropein, a bitter glucoside present in the pulp of olive fruits, has antimicrobial activity has also been made by several workers (*6, 13, 14, 28, 29, 30*) although others have been unable to demonstrate such activity (*8*). Indeed, Garrido-Fernandez and Vaughn (*8*) reported that oleuropein at concentrations of 0.2% (w/v) and 0.4% (w/v) could be utilised in basal medium by microorganisms associated with olive fermentations but only after a delay in growth of 2-5 days. Nychas *et al.* (*17*) demonstrated that the phenolic compounds extracted from olives were able to inhibit protein secretion by *S. aureus* and furthermore Tranter *et al.* (*35*) found that the enterotoxin B production was influenced by the addition of oleuropein in broths. It should be noted that there have been limited

studies on the effect of phenolic extracts and particularly of oleuropein on food poisoning bacteria or production of bacterial toxins in a model food system. The only relevant work in general with phenolics in a model food system is this with *Listeria monocytogenes* of Payne *et al.* (*21*).

More work can be done on the effect of oleuropein in combination with other factors such as pH or NaCl, particularly since its effect appears to be accentuated in the presence of 5% NaCl (*7*). Furthermore it has recently been reported that oleuropein retains activity even after heating at 121° C/15 min (*29*) which may not preclude its use in heat processed foods.
The aim of this work was 1. to establish whether there are interactive effect between inoculum size, pH and the concentration of oleuropein in the medium on the growth of *S. aureus* S-6 and 2. to determine the activity of olive phenolics in a model milk system against the growth as well as on enterotoxin B production by *S. aureus*

Material & Methods

Bacterial strain

Staphylococcus aureus S-6 (NCTC 10657), a producer of enterotoxin B, was maintained on slopes of plate count agar (Oxoid, Hampshire, UK) at 4°C and subcultured every two weeks. This organism was kindly provided by Dr. H.S Tranter (Public Health Laboratories, Porton Down, UK).

Growth media

NZ amine medium (NZA) containing (g/l): N-Z amine A (Sheffield Chemical Co. Norwich, N.Y , USA), 20.0 g; proteose peptone No.3 (Difco Laboratories, Detroit, MI) 20.0; was sterilised by autoclaving. Filter-sterilised (0.22μm, Millipore) growth factor solution (1% w/v niacin and 0.5% w/v thiamin) was added to each medium to give final concentration of 0.001% w/v niacin and 0.0005% w/v thiamin respectively and 1% (final concentration glucose added immediately prior to inoculation.

The model milk system consisted of a nonfat milk (kindly provided from Professor Ch. Kehagias, TEI, Athens, Greece). Solids suspension (10% w/v) was prepared by rehydrating the nonfat milk solids in deionised water and autoclaving at 115°C for 20 min. The milk was dispensed (2.5ml final volume) into sterile Malthus (Radiometer International, Copenhagen, Denmark) glass reaction cells as in the case of culture media described below.

Experimental procedure

The growth medium (NZA or milk) was dispensed (2.5ml final volume) into sterile closed glass reaction cells . Oleuropein (Extrasynthese, 69730 Genay, France) or phenolic compounds extracted from olives (*1, 33*) were dissolved in distilled water (250 mg/ml). The suspension was warmed up to 40-50°C to aid dissolution and the solution sterilised by filtration (0.22 μm; Millipore). This solution was added to reaction cells each containing growth medium (NZA or milk) to give a final concentration of 0, 0.1 and 0.6% oleuropein (for broth) or 0,0.5%,1%, 1.5% and 2% phenolics (for milk system) in an ultimate reaction volume of 2.5ml. These reaction cells were incubated at 37°C for 1h before inoculation to have the same temperature with that of the incubator and with that of the inoculum which will be used. The cells were then inoculated with 0.1ml of an overnight culture of *S. aureus* S-6 grown at 37° C in the same growth media (NZA). After inoculation the reaction cells were incubated statically in the water bath of a Malthus 2000 system maintained at 37°C. Changes in the conductance of the medium during growth were monitored every six minutes by the Malthus 2000. After 48h the reaction cells were sampled aseptically to determine the viable count and enterotoxin concentration.

Microbiological growth monitoring

Bacterial growth was monitored by conductance measurements using the Malthus 2000 instrument (Radiometer International, Copenhagen, Denmark). This system detects changes in conductance caused by bacterial metabolism in the growth medium of the reaction cells which contain platinum electrodes. The analyser monitors conductance changes every 6 mins and records the data. Changes are expressed in microsiemens (μS); they are shown graphically as conductance curves. The conductance detection time (DT), expressed in hours, can be considered to study the microbial metabolism also. It is defined as the time interval between the

start of conductance monitoring and the beginning of the acceleration phase of the signal *(5)*.

Viable count

Cultures samples (1ml) were serial diluted in 9 ml amounts of Ringer solution. Six 0.02 ml amounts of each solution were placed individually on the dried surface of plate count agar and the colonies counted after 24h at 37° C

Enterotoxin assay

The production of enterotoxin B in the presence or absence of phenolics was estimated by the reversed passive latex agglutination (RPLA) kit (Unipath Limited, Hampshire, UK). For this a sample (1ml) was removed from the reaction cells and centrifuged (10,000 x **g**) for 5 min to remove the cells. The supernatant fluid was removed and stored at -80°C prior to use. A portion of the defrost sample was added to latex beads coated with anti-enterotoxin B serum in wells in microtiter plates according to the manufacturer's instruction. In the first well for the titration of the enterotoxin B the sample was undiluted (*31*). Agglutination of the latex spheres was recorded as a positive reaction. Purified enterotoxin B (kindly supplied by H.S Tranter) was used as control as well as that included in the kit. Although the kit is not able to give an accurate quantitative measurement of the toxin present in culture fluids, the amount of toxin present was estimated according to the equation: staphylococcal enterotoxin B / ml of original solution = detection limit x 2^{n-1}, where the detection limit according to the manufacturer is 0.5ng and n is the number of the well in which the endpoint of a given sample is found (*31*). According to Park and Szabo (*19*)

the kit shows a high specificity and sensitivity with a detection limit of O.75ng enterotoxin/g food. However Tranter and Brehm (*34*) have reported that this test is sufficiently sensitive to detect 1-2ng/ml. Moreover the SET-RPLA kit did not show any non specific reaction in either phosphate-buffered saline or N-Z Amine A broth with or without oleuropein before inoculation with the organism.

Phenolic compounds tested

Oleuropein (80% pure according to manufacturer) was obtained from Extrasynthese (69730 Genay, France). The questionable purity of oleuropein was also reported from Tassou *et al.* (*32*) and Tassou (*33*).
Phenolics from olives were used also. Olives (200g) were frozen in liquid nitrogen and, after removal of the pits, crushed in a mixer (Kenwood chef). The mixture was immediately homogenized in 80% (v/v) ethanol in water. After agitation at 4°C for 20 min followed by filtration (Whatman no 1), the residue was again extracted with 80% ethanol. Aqueous- alcohol extracts were collected and ethanol was evaporated under vacuum. Four succesive petroleum ether extractions removed pigments and most of the lipids. The phenolic compounds were then extracted with ethylacetate. After three succesive extractions, the ethylacetate was removed under vacuum and the dry residue (0.5gr) dissolved in 10ml water (*33*)

Statistical analysis

In the case of the study of inhibition in broth a three way analysis of variance experiment was designed. Four pH levels 5, 6,7 and 8, three inoculum sizes ($\log_{10}$ 8.15 cfu/ml, $\log_{10}$ 7.15 cfu/ml, $\log_{10}$ 6.15 cfu/ml), and

three concentrations of oleuropein (0%, 0.1% and 0.6%) were studied in two different experiments in duplicate samples for each treatment. The final growth (expressed as microsiemens) and the detection time (expressed as hours) of this organism were collected from the reaction cells (144 in total) of the Malthus Instrument and analysed by analysis of variance with the SYSTAT (Systat Inc., Evanston, IL, USA).

The effect of the addition of phenolic extract (0, 0.5, 1, 1.5 & 2%) of olives on *S. aureus* in milk system was studied in two different experiments. In each experiment 8 reaction cells were used for each treatment. The final viable counts data were analysed with SYSTAT also (analysis of variance one way, F-test).

Results

The size of initial inoculum, the amount of oleuropein and the pH of the medium influenced the growth of *S. aureus* (Table 1a,b). Indeed the three way analysis of variance showed that the final growth (in microsiemens) or the detection time (hours) were affected by all the above mentioned factors (Table 2).

In particular the presence of oleuropein at the time of inoculation resulted in an increase in the lag phase (detection time) and in a decrease in growth phase (final growth) of *S. aureus*.
These effects appeared to be concentration dependent (Table 1a,b). Growth was inhibited by higher concentrations of oleuropein (0.6%) especially at low pH (pH 5 and 6) and with low inoculum size, while lower concentrations of oleuropein (0.1%) delayed mainly the onset of the growth whilst final growth of the organism was variable (Table 1a,b).

The final growth of *S. aureus* was higher at pH 7 and 8 than at pH 5 and 6 in samples inoculated with the same inoculum and at the same concentration of oleuropein (Table 1a). When the detection time was analysed (Table 1b) it was found that this was higher in broths with pH 5 and 6 than that in broths with pH 7 and 8, inoculated with the same inoculum and at the same concentration of oleuropein.
In a few samples with the lowest inoculum size, the Malthus system gave detection time very close to the end of the experiment, mainly in the presence of higher concentrations of oleuropein (Table 1). Although there was a small increase in the conductivity (not greater than 60μS) of these samples over 48h there was clearly no observable growth (determined by viable counts) in these tubes, in comparison with the initial inoculum size used in these cells. As long detection times were occasionally recorded in

uninoculated medium such observations may have been the result of small fluctuations in the medium baseline.

Staphylococcus aureus inoculated in sterile reconstituted milk grew extensively as it is shown in Fig 1. When 0.5% (w/v) of olive extract was added the detection time was delayed by almost 3 hours but there was no influence on the growth or conductance curve as noted in Malthus system (Table 3 and Fig. 1). The concentration of 1% delayed the detection time to almost 11h (Table 3) while 1.5 and 2% olive extract showed a significant inhibition as evidenced by long detection times and final height of the conductance curves (Fig. 1). Although there was complete inhibition of microbial activity in high concentrations of olive extract, no appreciable differences in the viable counts were noted (Table 3). Indeed the viable counts in the case of 2% extract was decreased about 1.5 $\log_{10}$ less than that of the control. Enterotoxin B production was more easily affected by the presence of olive extract. About half the amount of toxin recovered in the control was detected in samples containing 0.5% or 1% olive extract. Moreover it was found to be very low with higher concentrations, 1.5 and 2% (Table 3) of phenolics.

Discussion

The antimicrobial action of phenolic antioxidant such as butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) against *S. aureus* is well established (*3, 4, 21, 22*). In contrast the antimicrobial action of naturally occurring phenolic compounds, which are widespread in nature, have not been investigated in great detail (*37*). Moreover many of these studies involved purified compounds evaluated in microbiological media (*10, 35, 36*). In the case of phenolics from olives, Rodriguez and his collaborators (*26*) concluded that at present their antibacterial effect cannot be correlated with a particular component of olive extract. Indeed the correlation of antimicrobial action with oleuropein is also difficult because of the questionable purity of the material used, at least from those who have checked it, in previous studies (*26, 32, 35*).

In this study NZA growth medium was selected as the standard medium since it gave greater conductance changes (maximum 400μS) for *S. aureus* in comparison to brain heart infusion (BHI). When these two media were compared, Nychas *et al.* (*18*) showed that while BHI contained higher amounts of proteins, NZA , a pancreatic digest of casein, contained higher concentrations of readily available nitrogen sources including amino acids, small peptides and ammonia. The availability of such compounds to the bacteria probably contributes to the greater change in conductance. This is due to the fact that the Malthus Instrument used in this study measures changes in electrical conductance in the microbiological media resulting from bacterial growth and accumulation of metabolic products (*5*). During growth, uncharged or weakly charged substrates (e.g proteins, carbohydrates) are converted into highly charged end products (eg amino acids, lactate etc) resulting in increased conductance of the growth medium.

The results presented in this study strongly suggest an inhibitory activity of oleuropein and the naturally occurring phenolics from olives against *S. aureus* S-6 in NZA broth and in a model milk system (Table 1 & 3; Fig.1). This was evident with the differences in detection time as well as in microbial activity as expressed with the changes in the conductance in the media.

Payne *et al.* (*21*) reported that the inhibitory action of propyl paraben, against *L. monocytogenes*, was not affected by the level of initial inoculum in the medium. In the present study, however, the growth of *S. aureus* in NZA medium, was related to initial inoculum size, initial pH and the concentration of oleuropein (Table 2). In this study it was found that in samples with pH 5 and low concentration of oleuropein (0.1%) the final growth of organism (Table 1) decreased with the decrease of the size of the initial inoculum. In contrast in samples with pH 6, pH 7 and pH 8 the final growth (expressed in μS) of this organism did not differ significantly. Similarly Tranter *et al.* (*35*) showed that while high concentrations (> 0.4%) of oleuropein inhibited growth of *S. aureus* S-6, low concentrations (0.1%) were shown to inhibit production of enterotoxin B in NZA medium (pH 7) despite little or no effect on final cell mass. They found that the pH of the media (even with the addition of the highest concentration of oleuropein) was similar to control (without addition of oleuropein) and further remained constant throughout the experiment. The decrease in the production of enterotoxin B from *S. aureus* in these samples could be attributed only to oleuropein as its presence prevented the production of lactate by assimilation of glucose which consequently did not alter the pH of the growth medium.

They also found that in BHI medium (pH 7) the low concentration (0.1%) of oleuropein did not affect growth or toxin production of this strain, possibly because of the presence of higher amounts of proteins which masked the

antimicrobial properties of these compounds (*27*). Nychas *et al* (*17*) demonstrated that extracts from olives could inhibit growth and protein secretion by *S. aureus* when added to NZA medium. Rico-Munoz and Davidson (*24*) found that the antimicrobial effect of BHA and TBHQ was influenced by the presence of different amounts of casein and corn oil. They reported that the increase of proteins in the media could influence the inihbitory effect of BHA and TBHQ against *S.aureus, Pseudomonas fluorescens* and *Saccharomyces cerevisiae*. In the presence of 3% casein, BHA caused less than one log cycle decrease in the growth of *S. aureus* while the same amount of BHA in higher concentrations of casein (6% and 9%) did not inhibit growth of this organism. Cornell *et al.* (*2*) reported that BHA was bound reversibly to casein, probably through hydrophobic interactions and decreased its antimicrobial action in nonfat dry milk system. They found that, at low concentrations, the amount of BHA bound was a linear function of concentration of free antioxidant. In the present study, binding of the phenolics by the milk protein could have been a cause for the loss in antimicrobial activity of extract, especially in low concentrations. Indeed this could account for the insignificant differences on the final viable counts found among the samples without or with very low concentration of extract (0.5%) (Table 3) compared to the final viable counts found in samples with higher (> 1%) concentration of extract. In contrast with the final growth (expressed in cfu/ml) of *S.aureus*, the amount of staphylococcal enterotoxin B produced in these samples differed significantly (Table 3) and were found to be influenced even by the addition, in the nonfat milk, of the lowest concentration of extract. Tranter *et al* (*35*) reported similar findings for the staphylococcal enterotoxin B production in BHI medium. Higher concentrations of extract compounds in milk, not only inhibited significantly the final growth of this organism but also affected drastically the amount of enterotoxin B production (Table 3).

Previous investigations with phenolic and/or antioxidant compounds have indicated that they cause leakage of constituents such as proteins, glutamate or potassium and phosphate from bacteria (*11, 14*). This may due to disruption of cell peptidoglycan (*28*) and/or damage of the cell membrane (*11*). According to Hugo (*11*) leakage is known to be a general phenomenon induced by many antibacterial substances. Prindle and Wright (*23*) noted that the mode of action of phenols is concentration dependent. Lower concentrations affect the activity of enzymes associated with energy production whilst high amounts cause a precipitation of proteins.

It needs to be noted that care should be exercised (eg by checking the viable count at the time of detection time or by monitoring the base line of an uninoculated sample in which all other parameters have been included) when interpreting detection times as well as values of final conductance of cultures grown in media which is not specifically designed for the Malthus Instrument (eg. NZA and reconstituted nonfat milk). In this study the increase in conductance either in NZA or in milk over the growth cycle was almost similar to that of identical cultures in medium (eg Special Peptone Yeast Extract) specially designed for this Instrument. This study has shown that the consistent effectiveness of phenolics (oleuropein and phenolic extract) against *S.aureus* S-6, in the broth culture and in the model milk system gives probably the basis for potential use of these antimicrobials in other dairy products. The ability of oleuropein to inhibit over a wide pH range (pH $<$ 7) gives it the potential to be used in different dairy products as a safeguard against proliferation of *S.aureus*. Moreover this study has also shown that automated conductance measurement can be used as a rapid and sensitive system for studing the effects of antimicrobial factors on toxin-producing bacteria in a model food system. The comparison of such methods of study over the classical ones have been reported from Giraffa *et*

al. (*9*) and Tranter *et al.* (*35*).

More information also needs to be gathered on the effects of these compounds on bacteria in combination with various environmental conditions such as temperature, pH and water activity in model food system.

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TABLE 1. Final growth[1] (a) and Detection time[2] (b) of *Staphylococcus aureus* S-6 with different inoculum size, pH and concentration of oleuropein.

Initial inoculum log_{10} cfu/ml	Concentration of oleuropein (%)	pH			
		5	6	7	8
-a-					
7.15	0	184.5[3]	245.5	326.0	336.0
	0.1	176.0	234.2	327.0	311.0
	0.6	128.4	165.2	304.0	336.0
6.15	0	229.0	232.2	340.4	324.0
	0.1	59.5	232.0	330.1	330.5
	0.6	91.5	151.8	298.0	75.1
5.15	0	127.3	269.0	320.0	309.0
	0.1	10.6	180.0	310.0	308.0
	0.6	38.6	143.0	292.0	62.4
-b-					
7.15	0	16.30[3]	15.10	4.10	3.75
	0.1	25.65	20.20	5.10	5.30
	0.6	25.85	26.15	6.65	11.15
6.15	0	21.50	16.50	5.50	5.65
	0.1	25.70	21.20	8.50	21.50
	0.6	34.70	26.10	12.65	27.10
5.15	0	25.60	22.00	9.10	8.75
	0.1	>48.00	14.60	10.60	25.20
	0.6	>48.00	26.10	19.40	>48.00

[1] expressed in microsiemens (μS)
[2] expressed in hours
[3] Each number is the average of four observations in two different experiments (two observations in each experiment)

TABLE 2. The effect of initial inoculum size, amount of commercial pure oleuropein added and the pH of the medium on the final growth (a) and on the detection time (b) of *Staphylococcus aureus* S-6 (Three way analysis of variance)

(a)

Source	df	f-ratio	Probability level
(A) Inoculum size	2	60025	0.0000
(B) oleuropein	2	100435	0.0000
(C) pH	3	331088	0.0000
A x B	4	11881	0.0000
A x C	6	16386	0.0000
B x C	6	22572	0.0000
A x B x C	12	12652	0.0000

(b)

Source	df	f-ratio	Probability level
(A) Inoculum size	2	43643	0.0000
(B) oleuropein	2	28978	0.0000
(C) pH	3	35314	0.0000
A x B	4	4501	0.0000
A x C	6	9514	0.0000
B x C	6	17349	0.0000
A x B x C	12	15035	0.0000

Inoculum size levels : $\log_{10}$ 7.15, $\log_{10}$ 6.15 and $\log_{10}$ 5.15 cfu/ml
Oleuropein concentration: 0, 0.1, 0.6% (w/v)
pH : 5, 6, 7, 8

TABLE 3. The effect of the addition of phenolic extract of olives on growth and enterotoxin B production from *Staphylococcus aureus*[1] S-6 in a model food system at 37°C after 48h of incubation

	% Extract	Detection time (h)	Final viable counts log_{10} cfu/ml	Enterotoxin B production ng/ml
	0	5.5[2]	9.04[2]a	200.0[2]
	0.5	8.2	9.10a	100.0
milk	1	16.5	8.60b	100.0
	1.5	26.8	8.20c	5.0
	2	26.8	7.50d	0.5

[1] Initial inoculum size log_{10} 7 cfu/ml
[2] Mean from two different experiments (8 reaction cells each experiment)

means with the same letter are not significantly different at 0.01 level using Duncan's multiple range test

Fig. 1 The effect of addition of olive extract on the growth of *Staphylococcus aureus* in a model food system (milk) at 37° C